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MOLECULAR STUDIES OF THE PICHINDE VIRAL GENOME

BY

(C) ARLENE INDRA RAMSINGH

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Molecular Studies of the Pichinde Viral Genome

submitted by Arlene Indra Ramsingh in partial fulfilment for the degree of Doctor of Philosophy

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To my parents - Sheila and Isaac,
my sisters especially Nal and Paddy and
my brothers.

To Serena Anne and Héléna Marie.

ABSTRACT

Pichinde virus, a member of the Arenaviridae, is a negative-strand RNA virus with a segmented genome. The aim of this research project was to define the Pichinde viral genes and their corresponding gene products. A prerequisite of this objective is an understanding of the structural organization of the viral genome.

The first objective was to determine the number of viral RNA species that comprised the Pichinde viral genome and to estimate the molecular weights of these RNAs by gel electrophoresis under various denaturing conditions. Different denaturing systems i.e. 10mM methylmercury hydroxide, glyoxal and DMSO and 2.2M formaldehyde were used to ensure the validity of the MW estimates. The genetic information of Pichinde virus appears to reside in 2 RNA segments, L and S. Two additional RNA species, 1.9kb (MW 0.6 X 10^6) and 3.1kb (MW 1.0 X 10^6) were detected in purified virus preparations when virus was harvested late (72 - 120 hr post infection) or when virus was harvested after a relatively high multiplicity of infection (1.0 pfu/cell). These 2 RNA species do not appear necessary for the initiation of viral infection since viral preparations lacking these RNA species were infectious.

The average estimates of the MW of the S and L RNAs are 1.26 X 10^6 and 2.83 X 10^6, respectively, using 10mM methylmercury hydroxide as the denaturant and 1.31 X 10^6 and 2.63 X 10^6,

respectively, using glyoxal and DMSO as the denaturant. The average MW estimates for the L and S RNAs using these 2 denaturants are thus in good agreement.

The next objective was to determine the gene coding capacity of Pichinde virus. The gene coding capacity would be different if both RNAs contain unique sequences or if the S RNA is a subset of the L RNA. The nucleotide sequence relationship between the L and S RNAs was examined by synthesizing cDNA from individual L and S RNAs for use as molecular probes in nucleic acid hybridization experiments. The conditions for cDNA synthesis were optimized for these experiments. About 89% of the L cDNA hybridized to the L RNA and 90% of the S cDNA hybridized to the S RNA. About 3% to 5% hybridization was observed when either L cDNA was hybridized to S RNA or when S cDNA was hybridized to L RNA. These results suggest that the L and S RNAs are mainly unique but that they do share a few common sequences.

The next objective was to assign the viral genes to the L and S RNAs. This task was approached from three different levels of increasing resolution. Having shown that the viral genetic information resides on the L and S RNAs, that the MWs of the L and S RNAs are 2.63-2.83 X 10^6 and 1.26-1.31 X 10^6 respectively and that the L and S RNAs contain different genetic information, one could deduce the potential coding capacity of each viral RNA species. Using this data, the 3 primary gene products (NP, pGP-C, L), detected by Harnish et al. (1981), can be assigned to the L or S RNA. Both NP and pGP-C can be mapped to the S RNA while the L protein is mapped to the L RNA. The second approach involved the use of a recombinant virus, Re-2 (Leung et al., 1981; Harnish et al., 1983). Analyses of the viral RNAs and proteins of the recombinant virus and the parental viruses substantiated the hypothesis that the L RNA encodes the L protein while the S RNA encodes the NP and pGP-C. The next aim was to determine the orientation of the NP and pGP-C genes on the S RNA and to locate the gene(s) at the nucelotide sequence level. A synthetic peptide corresponding to the first 8 amino acids encoded in the 3' proximal end of the S RNA was coupled to BSA. Antiserum to this coupled peptide defined the product encoded in the 3' proximal end of the S RNA as the NP. This approach also identifies the initiation site of translation of the NP at the first UAC triplet (nucleotide residues 84-86) from the 3' terminus of the S RNA. Since both the NP and pGP-C genes map on the S RNA and the NP gene resides in the 3' proximal end of the S RNA then by deduction, the pGP-C gene should reside in the 5' proximal end of the S RNA.

The next objective was to determine the nucleotide sequence relationships among the various viral RNA species i.e. L, S, 3.1kb and 1.9kb. Molecular probes in the form of cDNA clones were constructed. RNA from purified virus preparations containing the L, S, 28S and 18S RNAs was used as the template for cDNA synthesis. Double-stranded DNA was cloned into the Pst 1 site of the plasmid vector, pBR322. Clones were screened by antibiotic sensitivity and by the size of their plasmid DNA. The resulting 21 cDNA clones segregated into 2 classes i.e. (17) virus-specific clones and (4) ribosomal-specific clones. The 17 virus-specific cDNA clones further segregated into 11 L clones (pPL1 to pPL11) and 6 S clones (pPS1 to pPS6). Four of the S clones, pPS1 to pPS4, were derived from a 1.9kb region at the 3' proximal end of the S RNA.

RNA blot analyses showed that there is sequence homology among all 4 RNA species i.e. L, S, 3.1kb and 1.9kb. These RNAs share some common sequences internally and at their 3' termini. The origin of the 3.1kb RNA is unclear. However, the 1.9kb RNA shares extensive homology with the 3' proximal half of the S RNA and may represent a truncated S RNA containing the NP gene.

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TABLE OF CONTENTS

CHAPTER PAGE

1 AN OVERVIEW OF ARENAVIRUSES. 1

I. Introduction 1

II. The Arenavirus Group 2

A. Members of the arenavirus group. 2

B. Historical considerations. 4

III. The Structure of Arenaviruses. 6

A. Morphology and size. 6

B. Biophysical properties 9

C. Nucleic acid composition 9

D. Protein composition. 16

E. Virus-associated enzymes 21

IV. Replication of Arenaviruses In Vitro 22

A. Cytopathogenicity. 23

B. Growth cycle 24

C. Viral persistence in vitro 27

D. Transcription and replication of viral RNA 30

E. Viral antigens 32

F. Viral replication and host functions. 34

V. Replication of Arenaviruses In Vivo. 35

CHAPTER PAGE

2 MATERIALS AND METHODS 39

I. Tissue Culture. 39

II. Pichinde Virus. 40

A. Preparation of stock virus. 40

B. Plaque assay. 40

C. Purification of virus and extraction of viral RNA 41

D. Preparation of labelled viral RNA 42

III. Vesicular Stomatitis Virus. 43

A. Purification of virus and extraction of viral RNA 43

B. Preparation of labelled viral RNA 43

IV. Cellular Ribosomal RNA. 44

A. Extraction of cellular ribosomal RNA 44

B. Preparation of labelled cellular ribosomal RNA 45

V. Estimations of the Molecular Weights of Pichinde Viral RNAs by Gel Electrophoresis under Different Denaturing Conditions 45

A. Agarose gel electrophoresis in 10mM methylmercury hydroxide. . . 45

B. Electrophoresis of RNA in composite polyacrylamide-agarose gels after denaturation in glyoxal and DMSO. 47

C. Agarose gel electrophoresis in 2.2M formaldehyde. 47

CHAPTER PAGE

VI. Preparation of Primer from Calf Thymus DNA. 48

VII. Determining the Optimum Conditions for the In Vitro Synthesis of Pichinde cDNA probes. 49

VIII. Isolation of Pichinde Virus L and S RNAs. 51

IX. Hybridization of [^{3}H] cDNA to RNA . . . 52

X. Preparation of Antiserum to Pichinde Viral Antigens. 52

XI. Preparation of a Synthetic Peptide Coupled to Bovine Serum Albumin . . . 53

A. Chemical synthesis of a Pichinde peptide [(Gly)^9-P(1-9) amide] . . 53

B. Purification of synthetic peptide 55

C. Preparation of covalently linked peptide - BSA complex 55

XII. Preparation of Antiserum to a Synthetic Peptide Coupled to Bovine Serum Albumin 56

XIII. Radioactive Labelling of Intracellular Proteins in Pichinde Virus-Infected Cells 57

XIV. Immunoprecipitation and Gel Electrophoresis of Viral Polypeptides. 57

XV. Molecular Cloning of the Pichinde Viral Genome. 58

A. Synthesis of double-stranded Pichinde cDNA from RNA extracted from purified virus 58

CHAPTER PAGE

B. Cloning of Pichinde cDNA into the Pst 1 site of pBR322 by homopolymer tailing. 60

C. Transformation of Escherichia coli LE392. 61

XVI. Purification of Plasmid DNA. 62

XVII. Preparation of Pichinde cDNA Probes. . . . 63

XVIII. Dot Hybridization. 63

XIX. RNA Blot Analyses. 65

XX. Preparation of a 3' End-Specific S RNA Probe. 65

3 SIZE ESTIMATION OF PICHINDE VIRAL RNA BY GEL ELECTROPHORESIS UNDER DIFFERENT DENATURING CONDITIONS. 67

I. Introduction. 67

II. Results 68

A. Agarose gel electrophoresis of Pichinde viral RNA in 10mM methylmercury hydroxide 68

1. Determination of the number of genomic RNA species present in purified Pichinde virus 68

2. Estimation of the molecular weight values of Pichinde viral L and S RNAs. 72

3. Determination of the molar ratios of Pichinde L and S RNAs. 73

B. Analysis of Pichinde viral RNA on polyacrylamide-agarose gels after denaturation with glyoxal and DMSO. 78

CHAPTER PAGE

C. Agarose gel electrophoresis of Pichinde viral RNA in 2.2M formaldehyde. 81

III. Discussion 84

4 DETERMINATION OF THE RELATIONSHIP BETWEEN THE L AND S RNAs OF PICHINDE VIRUS BY HYBRIDIZATION TO cDNA PROBES. 89

I. Introduction 89

II. Results. 90

A. Generation of random DNA primers for use in cDNA synthesis. 90

B. Conditions for cDNA synthesis. 94

C. cDNA synthesis from individual L and S RNAs 102

III. Discussion 106

5 GENE MAPPING OF THE PICHINDE VIRAL GENOME . . . 109

I. Introduction 109

II. Results. 110

A. Gene assignment based on the potential coding capacity of the viral genome 110

B. Orientation of the genes on the S RNA. 114

1. Immunoprecipitable polypeptides in BHK cells 116

2. Immunoprecipitation of BHK cell extracts using an antiserum against a synthetic Pichinde peptide coupled to BSA 118

III. Discussion 118

CHAPTER PAGE

6 CONSTRUCTION OF RECOMBINANT cDNA PROBES FOR THE STUDIES OF GENOME STRUCTURE IN PICHINDE VIRUS. 124

I. Introduction. 124

II. Results 125

A. Identification of cDNA clones 125

B. Determination of the specificity of the cDNA clones by hybridization studies 125

C. Assignment of the Pichinde virus-specific cDNA clones to their appropriate genomic L or S RNA. . . . 130

D. Orientation of the clones on the S RNA 135

III. Discussion. 138

7 SUMMARY 145

REFERENCES 147

LIST OF TABLES

TABLE	DESCRIPTION	PAGE
1	Members of the Arenavirus group.	3
2	RNA species isolated from arenaviruses	11
3	Estimated molecular weights (X 10^3) of proteins found in arenaviruses	17
4	Summary of MW determinations of Pichinde viral L and S RNAs after gel electrophoresis in 10mM methylmercury hydroxide.	76
5	Molar ratios of the L and S RNAs in an early harvest (24 to 72 hr post infection) of purified Pichinde virus.	79
6	Summary of MW determinations of Pichinde viral L and S RNAs after denaturation in glyoxal and DMSO followed by electrophoresis in composite polyacrylamide-agarose gels.	83
7	Estimation of the potential coding capacity of the Pichinde viral genome	111
8	Assignment of the polypeptides to the Pichinde viral genome on the basis of size.	112
9	Assignment of the Pichinde virus-specific cDNA clones to their genomic RNAs (L or S) by dot hybridization using cDNA probes prepared from isolated L or S RNA.	131
10	Summary of hybridization data generated by RNA blot analysis.	134

LIST OF FIGURES

FIGURE PAGE

1a Ethidium-bromide stained gel profile of Pichinde viral RNA (obtained from virus harvested 24-72 hr post infection) after electrophoresis in 1% agarose containing 10mM methylmercury hydroxide. 69

1b A stained gel profile of Pichinde viral RNA extracted from virus harvested 24 to 120 hr post infection (multiplicity of infection: 1 pfu/cell). 69

2 Electropherograms of [^{3}H]-uridine-labelled Pichinde viral RNA and [^{32}P]-labelled BHK ribosomal RNA after co-electrophoresis in a 1.5% agarose gel after denaturation in 10mM methylmercury hydroxide. 70

3 Electropherogram of [^{3}H]-uridine-labelled VSV RNA in a 1.5% agarose gel after denaturation in 10mM methylmercury hydroxide. . 74

4 Log of the MW versus mobility plots for Pichinde viral RNAs after electrophoresis in 10mM methylmercury hydroxide at 4 different agarose concentrations (0.75 , 1.00, 1.25 and 1.50%). 75

5 Ferguson plots for Pichinde L and S RNAs, 28S ribosomal RNA and VSV RNA where log mobility is plotted as a function of the agarose concentration 77

6 Acridine-orange stained gel profile of Pichinde viral RNA and VSV RNA after denaturation in glyoxal and DMSO followed by co-electrophoresis in a 1% composite polyacrylamide-agarose gel. . 80

7 Log of the MW versus mobility plots for Pichinde viral RNAs after denaturation in glyoxal and DMSO followed by electrophoresis in composite polyacrylamide-agarose gels. . . . 82

FIGURE PAGE

8 Gel filtration profile of oligodeoxynucleotides after fractionation on a Sephadex G-50 column. 91

9 Effect of different size classes of oligodeoxynucleotides on *in vitro* cDNA synthesis 92

10 Effect of oligodeoxynucleotide primer-to-RNA template ratio on *in vitro* cDNA synthesis . . . 93

11 Effect of KCl concentration on cDNA synthesis 95

12 Effect of DTT concentration on cDNA synthesis 96

13 Effect of BSA concentration on cDNA synthesis 98

14 Effect of actinomycin D concentration on cDNA synthesis. 99

15 Effect of template RNA concentration on cDNA synthesis. 100

16 Effect of reverse transcriptase concentration on cDNA synthesis 101

17 Effect of various concentrations of cold dCTP on cDNA synthesis 103

18 Hybridization of $[^3H]$-L cDNA with Pichinde viral L or S RNA. 104

19 Hybridization of $[^3H]$-S cDNA with Pichinde viral L or S RNA. 105

20 Nucleotide sequence of the 3' terminus of the S RNA of Pichinde virus 115

21a PAGE analysis of immunoprecipitated Pichinde virus-specific polypeptides from BHK cells. . . 117

21b PAGE analysis of immunoprecipitated Pichinde virus-specific polypeptides from BHK cells. . . 117

22 Mapping of the Pichinde viral genome. 121

FIGURE PAGE

23 Screening of recombinant plasmid DNAs by size using agarose gel electrophoresis. 126

24 Identification of Pichinde virus-specific clones by dot hybridization 128

25 Agarose gel electrophoresis of Pst 1-digested recombinant plasmid DNAs 129

26 Identification of L and S clones by RNA blot analyses 133

27 Generation of a 3' end-specific S RNA probe 136

28 Autoradiogram of 3' end-labelled S RNA preparations after alkali digestion for 0, 15, 30 and 45 min followed by electrophoresis in agarose containing 10mM methylmercury hydroxide 137

29 Identification of S clones that map within the 3' proximal end of the S RNA by dot hybridization 139

30 Generation of the 1.9kb RNA via two possible mechanisms. 144

CHAPTER 1

AN OVERVIEW OF ARENAVIRUSES

I. Introduction

The first arenavirus to be isolated, almost 50 years ago, was lymphocytic choriomeningitis (LCM) virus (Armstrong and Lillie, 1934). However, only in the last 15 years have we been able to gain some understanding of the biology of these viruses and their biochemical and biophysical properties.

Research efforts to gain a better understanding of the arenaviruses stem from two major concerns. Four members of the arenavirus group, LCM, Junin, Machupo and Lassa viruses, cause severe and sometimes fatal disease in man, therefore efforts have been made to acquire knowledge which could be helpful in preventing or treating these diseases (Rawls and Leung, 1979). Secondly, arenaviruses characteristically produce persistent infections in their natural hosts. These viruses therefore readily lend themselves to studies on viral persistence. The phenomenon of viral persistence also has clinical significance since persistent virus infections seem to play a role in the etiology of certain chronic or degenerative diseases in man. The underlying mechanisms which allow viruses to persist in their hosts are currently unknown. Arenaviruses are eminently suitable for studying viral persistence since these viruses can establish persistent infections both _in vivo_ and _in vitro_. The persistent infections established _in vitro_ closely resemble those _in vivo_ e.g. LCM virus in L cells (Lehmann-Grube, 1967; Staneck _et al_.,

1972) and Pichinde virus in BHK cells.

This overview attempts to describe the knowledge on arenaviruses pertinent to this thesis project. Although information has been obtained on the structural organization of these viruses, many fundamental questions on their mode of replication, both *in vivo* and *in vitro*, remain unanswered.

This thesis project focused on the physical and genetic nature of the genome of Pichinde virus, as well as the relationship between the viral genes and their corresponding gene products. The resulting knowledge should aid in a better understanding of the structure and replication of arenaviruses.

II. The Arenavirus Group

A. Members of the arenavirus group

The taxonomic group, Arenaviridae, was proposed primarily on the basis of similarity in virus structure, RNA content and a group-specific antigen that was detected by immunofluorescence (Rowe *et al*., 1970). The name arenavirus derives from the Latin word "arena" (sand) and reflects the characteristic fine granules seen within the viruses by electron microscopy. The Arenaviridae currently consists of 11 different viruses; the Old World species include LCM virus, Lassa virus and Mozambique virus, the New World species include members of the Tacaribe complex i.e. Junin, Tacaribe, Machupo, Amapari, Parana, Tamiami, Pichinde and Latino viruses. The arenaviruses, their distribution and principal vertebrate hosts are outlined in Table 1.

Table 1: Members of the Arenavirus group

VIRUS	DISTRIBUTION	PRINCIPAL VERTEBRATE HOST	REFERENCES
OLD WORLD SPECIES:			
LCM	Worldwide	*Mus musculus*	Armstrong and Lillie, 1934.
Lassa	West Africa	*Mastomys natalensis*	Monath *et al*., 1974.
Mozambique	Mozambique	*Mastomys natalensis*	Wulff *et al*., 1977.
NEW WORLD SPECIES:			
Tacaribe complex			
Junin	Argentina	*Calomys laucha* *Calomys musculinus* *Akodon azarae*	Parodi *et al*., 1958.
Tacaribe	Trinidad	*Antibesis liturates* *Antibesis jamaicensis*	Downs *et al*., 1963.
Machupo	Bolivia	*Calomys callosus*	Johnson *et al*., 1965a.
Amapari	Brazil	*Oryzomys goeldi* *Neacomys guianae*	Pinheiro *et al*., 1966.
Parana	Paraguay	*Oryzomys buccinatus*	Webb *et al*., 1970.
Tamiami	Florida	*Sigmodon hispideis*	Calisher *et al*., 1970.
Pichinde	Colombia	*Oryzomys albigularis*	Trapido and Sanmartin, 1971.
Latino	Bolivia	*Calomys callosus*	Johnson *et al*., 1973.

B. Historical considerations

LCM virus, the prototype of the arenavirus group, was initially isolated in 1933 from a monkey used for the passage of what was then thought to be St. Louis encephalitis virus (Armstrong and Lillie, 1934). Shortly thereafter, the virus was isolated from five patients with aseptic meningitis (Rivers and Scott, 1935). Subsequent studies established the house mouse (_Mus musculus_) as the principal reservoir of LCM virus (Lepine _et al._, 1937; Armstrong and Sweet, 1939). Although house mice may harbour LCM virus, this virus seems to have minor clinical significance in man since most patients with LCM virus infections recover (Murphy, 1977). Not long after the virus was isolated, virus persistence associated with congenital and neonatal infection of mice was recognized (Traub, 1936). LCM virus was thus extensively studied from an immunological viewpoint and has provided much information in the area of immune tolerance.

In 1958, the etiological agent of Argentine hemorrhagic fever, Junin virus, was isolated (Parodi _et al._, 1958) and found to be serologically related to another virus, Tacaribe, which had been recovered from fruit-eating bats in Trinidad (Mettler _et al._, 1963; Downs _et al._, 1963). In 1963, Machupo virus, the causative agent of Bolivian hemorrhagic fever, a disease indistinguishable from Argentine hemorrhagic fever, was isolated and shown to be related to Junin and Tacaribe viruses (Johnson _et al._, 1965a; Johnson _et al._, 1965b). It was then clear that a new virus group could be defined. Junin, Tacaribe and Machupo viruses were grouped into what became known as the Tacaribe complex which was subsequently expanded with the isolation and identification of Amapari virus (Pinheiro et al., 1966), Parana virus (Webb et al., 1970), Tamiami virus (Calisher et al., 1970), Pichinde virus (Trapido and Sanmartin, 1971) and Latino virus (Johnson et al., 1973).

The first suggestions that the viruses of the Tacaribe complex might be related to LCM virus, came from observations that the virus-host interactions were similar. Machupo virus induced a chronic tolerant infection in its natural rodent host (Johnson et al., 1973), a phenomenon similar to that observed with LCM virus. Shortly after the report on the morphological characteristics of LCM virus (Dalton et al., 1968), Murphy et al. (1969) showed that Machupo virus shared the same structural properties, including the presence of host cell ribosomes within virions.

Murphy et al. (1970) later showed that all members of the Tacaribe complex shared the morphological characteristics of Machupo virus and LCM virus. Thus a new family of viruses, incorporating LCM virus and the Tacaribe complex, known as arenaviridae was created. This group was expanded to include Lassa virus which was first isolated from the blood of a missionary nurse from Jos, Nigeria (Buckley and Casals, 1970; Frame et al., 1970). Mozambique virus was subsequently isolated from *Mastomys* natalensis and shown to have a close antigenic relationship with Lassa virus (Wulff et al., 1977). It seems that the arenavirus group will expand when more virus studies of rodent populations in different areas of the world are undertaken (Rawls and Leung, 1979).

III. The Structure of Arenaviruses

A. Morphology and size

All members of the arenavirus group share similar morphological features. These viruses consist of dense, well defined membrane envelopes with closely spaced projections and unstructured interiors containing a variable number of electron-dense granules (Pfau, 1974). Discrete nucleocapsid structures, seen in most viruses, are not found in arenaviruses.

The initial report on the structure of arenaviruses came from Dalton and co-workers in 1968. They reported that LCM viruses are pleomorphic in shape and range in size from 50 to 300nm in diameter with an average diameter of 110-130nm. The individual surface projections or spikes are 5-10nm in length and club shaped (Murphy and Whitefield, 1975; Welsh et al., 1976; Gard et al., 1977; Vezza et al., 1977). When examined in cross-section perpendicular to the long axis, these projections appear hollow (Murphy and Whitefield, 1975). The electron-dense granules which closely resemble host cell ribosomes are 20-30nm in diameter and these granules disappear after ribonuclease treatment (Dalton et al., 1968). Later biochemical analyses revealed that indeed, ribosomes can be isolated from purified virus preparations. The ultrastructure of LCM virus has been repeatedly confirmed (Murphy et al., 1969; Murphy et al., 1970; Ofodile et al., 1973).

Murphy et al. (1969) showed that there is a striking morphological similarity between LCM, Machupo and Tacaribe viruses grown in vivo or in vitro. These similarities were also observed in Latino, Parana, Pichinde and Tamiami viruses (Murphy et al., 1970) and later in Lassa virus (Speir et al., 1970).

The morphology of the nucleocapsid structure of three arenaviruses has been described. Negative contrast electron microscopy of spontaneously disrupted Pichinde virus showed that the nucleocapsids appear as convoluted filamentous strands (Vezza et al., 1977). The strands were reported to be 450nm in length and 9-15nm in diameter. Young et al. (1981) have shown that the nucleocapsids of Pichinde virus appear as strands with a beaded appearance. These beads or nucleosomes are 3 to 4nm in diameter and are spaced 5 to 7nm apart. Gentle disruption of the virus shows that the nucleosome strands are organized as helical structures 10 to 12nm in diameter. Nucleocapsids appeared both as circles and "spider-forms". The latter configuration suggested to the authors that the virion nucleocapsid is packaged into a discrete core structure. The nucleocapsid structures of Tacaribe and Tamiami viruses appear similar to that of Pichinde virus (Palmer et al., 1977; Gard et al., 1977). Nucleocapsids appeared as closed circles with two predominant length classes of 640 and

1300nm. The two length classes had no direct relationship to the sizes of the viral RNAs. The circular nucleocapsid structures are not typical helices as reported for the nucleocapsids of other RNA viruses. Ribosomes were not associated with the nucleocapsid structures. The morphology of the nucleocapsids of arenaviruses is not unique since similar observations have been made with the bunyaviruses (Obijeski and Murphy, 1977).

The ribonucleoproteins of arenaviruses are sensitive to RNase digestion. The predominant protein species found in this structure is a 66,000 MW polypeptide in Pichinde virus (Buchmeier et al., 1977) and a 63,000 MW polypeptide in LCM virus (Buchmeier et al., 1978). Gard et al. (1977) reported a minor polypeptide of 79,000 along with the major polypeptide of 68,000 in ribonucleoprotein structures of Tacaribe and Tamiami viruses. An RNA transcriptase activity was found associated with the ribonucleoprotein structure of Pichinde virus (Leung, 1978; Leung et al., 1979).

The arenaviruses appear to mature by budding in a manner similar to that of other enveloped RNA viruses. This process seems to be the same for virus replicating in vitro and in vivo. Budding of progeny virus occurs at the plasma membrane. Membrane changes detectable by electron microscopy occur at the site of virus formation (Mannweiler and Lehmann-Grube, 1973; Murphy and Whitfield, 1975). In addition to these changes, alterations in the distribution of intracytoplasmic ribosomes have been reported

Abelson et al., 1969, Murphy et al., 1970; Manweiler and Lehmann-Grube, 1973; Murphy and Whitfield, 1975). Large aggregates of ribosomal-like structures are seen scattered throughout the cytoplasm of LCM virus-infected L cells (Manweiler and Lehmann-Grube, 1973). These intracytoplasmic inclusion bodies are found in cell cultures and in animal hosts and appear to be composed of masses of ribosomes embedded in a matrix of virus-specific proteins (Abelson et al., 1969).

B. Biophysical properties

The stability and physical properties of the arenaviruses have been reviewed by Pfau et al. (1974) and Rawls and Buchmeier (1975). As expected for enveloped viruses, the arenaviruses are sensitive to lipid solvents such as ether, chloroform and sodium deoxycholate. Arenaviruses rapidly lose infectivity below pH 5.5 and above pH 8.5 and are relatively heat-sensitive. This sensitivity can be reduced by the addition of protein to the medium. The buoyant densities of these viruses are 1.17-1.18 g/cm^3 in sucrose gradients, 1.18-1.20 g/cm^3 in cesium chloride gradients and 1.14 g/cm^3 in amido triazole gradients (Gschwender et al., 1975). The reported sedimentation velocities for LCM virus varied from 76S (which could be an artefact) to 470-500S. A value of 300-325S was obtained for Pichinde virus.

C. Nucleic acid composition

The arenaviruses can be described as enveloped, negative-strand RNA viruses with segmented genomes. Definitive proof of the RNA content of arenaviruses was obtained after analyses of purified LCM and Pichinde viruses (Pedersen, 1970, Carter _et al._, 1973). Nucleic acid was released from purified virus by treatment with sodium dodecyl sulfate and separated by centrifugation in sucrose density gradients into three peaks. These peaks disappeared if, prior to sedimentation in sucrose gradients, the isolated nucleic acid was treated with RNase but not with DNase. Furthermore, as with single-stranded nucleic acids, the sedimentation rates of the 3 RNA species were dependent on the salt concentration in the sucrose gradients.

The number and size of RNA species extracted from purified virus preparations have been determined mainly by centrifugation in sucrose gradients and by polyacrylamide gel electrophoresis (PAGE) (Table 2). Based on these analyses, the RNA can be subdivided into four classes:

a) host cell ribosomal RNA i.e. 28S and 18S RNAs,
b) low-molecular weight RNAs i.e. 4-6S RNAs,
c) genomic RNA which consists of at least 2 RNA species, L (large) and S (small),
d) additional RNA species whose nature is unclear.

The evidence for the host cell origin of the 28S and 18S RNA comes from several experiments. Pedersen (1971) showed that the RNA isolated from purified LCM virus was resolved into 4 bands, two of which coincided with 28S and 18S host cytoplasmic RNA. These two bands disappeared when RNA was examined from

TABLE 2

RNA species isolated from arenaviruses

Virus	Genomic RNA	Ribosomal RNA	Small RNA	Other RNA	References
LCM	L 31-34S (2.1 X 10^6) S 23-25S (1.1 X 10^6)	28S (1.7 X 10^6) 18S (0.7 X 10^6)	4S 5S 5.5S		Pedersen, 1971;1973
Pichinde	L 31S (2.1 X 10^6) S 22S (1.1 X 10^6)	28S (1.7 X 10^6) 18S (0.7 X 10^6)	4-6S		Carter *et al*., 1973
	L (3.2 X 10^6) S (1.6 X 10^6)	28S 18S			Vezza *et al*., 1977; 1978
	L 31S S 22S	28S 18S		15S	Farber and Rawls, 1975
	L 31S S 22S	28S 18S	4-6S	15S	Dutko *et al*., 1976
	L S	28S 18S		X (1.0 X 10^6) 20S? Y (0.5 X 10^6) 15S?	Dimock *et al*., 1982
Tacaribe	L S	28S 18S		20S? 15S?	Vezza *et al*., 1978
Junin	33S (2.4 X 10^6) 25S (1.34 X 10^6)	28S 18S	4S 5S 5.5S		Anón *et al*., 1976
Parana	37S 24S	28S 18S			Dutko *et al*.,1978

virus grown in cells treated with a concentration of actinomycin D that prevented synthesis of ribosomal RNA but did not block viral RNA synthesis. The 28S and 18S RNA species could be isolated from 60S and 40S ribosomal subunits, respectively, released from purified virus (Farber and Rawls, 1975; Pedersen and Konigshofer, 1976). The base composition and methylation ratios (Carter et al., 1973) and oligonucleotide fingerprint data (Vezza et al., 1978) of the 28S and 18S RNAs in Pichinde virus were similar to those of the host cell ribosomal RNAs. The relative amounts of 28S and 18S RNAs in some virus preparations could account for up to 50% of the total [^{3}H] uridine incorporated into the viruses (Pedersen, 1971; Carter et al., 1973). This proportion seems variable since relatively small amounts of 28S and 18S RNAs were detected in a heat-resistant clone of Pichinde virus (Vezza et al., 1978).

Although the evidence from morphological and biochemical studies indicates that host cell ribosomes are present within arenaviruses, the significance of this observation is unclear. Leung and Rawls (1977) examined the possibility that the virion ribosomes are involved in the initial phases of virus replication by using a ts cell mutant, with thermolabile 60S ribosomal subunits. Replication of Pichinde virus in these cells at the permissive temperature resulted in incorporation of the temperature sensitive ribosomal subunit. Virus containing the thermolabile 60S ribosomal subunit was able to replicate as well as virus containing wild-type ribosomes at the nonpermissive temperature.

These results suggest that virus-associated ribosomes are not required for arenavirus replication. However, these experiments do not exclude the possibility that the ribosomes play a role in the assembly of progeny virions.

Low-molecular-weight RNAs, sedimenting at 4S to 6S in sucrose gradients, have been observed in purified preparations of LCM, Pichinde and Junin viruses (reviewed by Pedersen, 1979). These RNAs could be resolved into 4S, 5S and 5.5S RNA species by PAGE (Pedersen, 1973; Anón et al., 1976). The size and methylation ratio of the 4-6S RNAs were similar to those of host cell transfer RNA. The 4-6S RNA species constituted approximately 6% and 7% of the total radiolabelled RNA in LCM virus and Junin virus respectively (Pedersen, 1973; Anón et al., 1976). It is therefore likely that the low-molecular-weight RNAs found in association with the arenaviruses, originate from RNA associated with host ribosomes that are incorporated into the virions (Pedersen, 1979).

All arenaviruses thus far examined contain at least 2 genomic RNA species i.e. L and S RNAs. These RNAs are of negative polarity since they lack the structural characteristics of messenger RNA such as the presence of capped and methylated structures at the 5' termini and polyadenylated sequences at the 3' termini. In addition, viral RNA could not be translated into viral proteins in an in vitro protein-synthesizing system (Leung et al., 1977). The L and S RNAs of Pichinde, LCM and Tacaribe viruses appear to contain different genetic information since their oligonucleotide fingerprint patterns are different (Vezza et al., 1978; Kirk et al., 1980; Dutko et al., 1981; Compans et al., 1981). These observations, if taken to indicate that the L and S RNA contain different genetic information, is in agreement with the high-frequency of genetic recombination obtained with temperature-sensitive Pichinde virus mutants (Vezza and Bishop, 1977).

The RNAs of arenaviruses appear to possess considerable secondary structure. Electron microscopic studies of non-denatured RNA isolated from Tacaribe, Pichinde and Tamiami viruses showed circular, linear and hairpin RNA forms. In addition, native RNAs are somewhat resistant to RNase digestion implying the presence of double-stranded regions (Vezza et al., 1978). Molecular weight estimates of the viral genomic RNAs have been obtained mainly on the basis of sedimentation coefficients and PAGE analysis of non-denatured or heat-denatured RNAs. The resulting estimates have varied from 2.1×10^6 to 3.2×10^6 and 1.1×10^6 to 1.6×10^6 for the L and S RNAs, respectively (Carter et al., 1973; Vezza and Bishop, 1977; Vezza et al., 1977). Since viral genomic RNAs have a high degree of secondary structure, valid MW estimations by gel electrophoresis require removal or reduction of secondary structure so that electrophoretic mobility becomes a function of MW. Stringent denaturation of RNA is usually accomplished with methylmercury hydroxide, glyoxal and dimethyl sulfoxide, formaldehyde or urea.

A more valid estimation of the MWs of the L and S RNAs would yield a better approximation of the coding potential of the arenavirus genome.

In addition to the two genomic RNA species, additional minor RNA species have also been reported in purified virus preparations. A 15S RNA species is sometimes seen in Pichinde virus preparations (Farber and Rawls, 1975; Dutko et al., 1976). A similar RNA species (MW 0.5-0.6 X 10^6) was observed in late harvests of Pichinde virus (Dimock et al., 1982), Tacaribe virus and Tamiami virus (Vezza et al., 1978) and also in LCM virus harvested from cells that had been infected with both standard and defective-interfering (DI) virus (Dutko, 1981). Another additional RNA species, designated 20S, (MW 1.0-1.1 X 10^6), migrating between the S RNA and the 18S rRNA during electrophoresis, has also been detected in the above-mentioned virus preparations and in Pichinde virus harvested from long-term persistent infections. The nature of the additional RNA species is unclear. What is the origin of these RNAs? Are they virus-specific? Are they related to the viral L and S RNAs? If so, what are the relationships? Are these RNAs associated with standard virus or with DI virus? Are these RNAs synthesized in acute or persistent infections? Do these RNAs appear concomitantly or at different times during viral replication? Could these RNAs be virus-induced but of cellular origin? If so, what is the role of the induced RNA(s)? Could these RNAs have regulatory roles in virus replication? These are just some of the questions that need to be answered before we can gain an understanding of the viral genome and how it functions.

D. Protein composition

The structural polypeptides of several arenaviruses have been examined and the results are summarized in Table 3. A common characteristic of these viruses is the presence of 2 or 3 major proteins. The nucleocapsid polypeptide, NP, is not glycosylated and molecular weight ranges of 60,000 - 72,000 have been reported for different viruses. Two glycoproteins, G1 and G2, have been detected in Lassa, Mozambique, LCM, Pichinde and Junin viruses. The reported molecular weights of G1 and G2 range from 44,000 - 72,000 and 34,000 - 40,000 respectively. However, only one glycoprotein size class with a molecular weight of 42-44K has been detected in Tacaribe and Tamiami viruses.

The location of the polypeptides in the virion has been examined by solubilizing the viruses with nonionic detergent followed by separation of the components by equilibrium centrifugation in CsCl gradients (Ramos _et_ _al_., 1972; Gard _et_ _al_., 1977; Vezza _et_ _al_., 1977) or in metrizamide gradients (Buchmeier _et_ _al_., 1978). The major non-glycosylated polypeptide, NP, was found tightly associated with the viral RNA. This polypeptide is therefore thought to be part of the ribonucleoprotein (RNP) complex. This idea was further substantiated by the results of Vezza _et_ _al_. (1977). They showed that polyethylene glycol-dextran

TABLE 3

Estimated molecular weights (X 10^{-3}) of proteins found in arenaviruses

Virus	Estimated MWs of Proteins							
	NP	G1	G2	G	High MW proteins	P protein	Other proteins	References
Lassa	72	52	39		115 (G)*			Kiley et al., 1981
Mozambique	72	54	40		115 (G)*		84 (G)*	Kiley et al., 1981
LCM	62	54	35				12	Buchmeier et al., 1978
LCM	67	55	39			75	91 25 12	Pedersen, 1973
Pichinde	66	64	38			77	12	Vezza et al., 1977
Pichinde	68	65	38			77	50 15	Gangemi et al., 1978
Pichinde	64	52	36		≈200		20 15	Harnish et al., 1981
Junin	60	44	34-39					Grau et al., 1981
Tacaribe	68			42		79		Gard et al., 1977
Tamiami	66			44		77		Gard et al., 1977

*G: glycosylated

phase extraction of detergent-disrupted Pichinde virus resulted in the NP separating with the dextran phase, a property common to ribonucleoprotein complexes. The nucleoprotein is the dominating structural protein of arenaviruses. It has been estimated by protein labelling studies that Pichinde virus contains 1530 molecules of NP per virion (Vezza et al., 1977) and this protein accounts for 70% of the protein label (Buchmeier et al., 1977; Vezza et al., 1977). The NP is also responsible for the serological cross-reactivity observed among the viruses of the Tacaribe complex (Buchmeier and Oldstone, 1978).

One to two glycoproteins have been detected in arenaviruses. The glycoproteins reside at the surface of the virion and are primarily the spike structures of the viral envelope. This conclusion is based on the following results. Treatment of Pichinde or LCM viruses with Pronase or bromelain (Vezza et al., 1977; Buchmeier et al., 1978) or of Tacaribe and Tamiami viruses with chymotrypsin (Gard et al., 1977) results in loss of spikes from the viral surface as seen by electron microscopy or by a change in the buoyant density of the virus particles. PAGE analysis of the spikeless virions showed a reduction in the amount of glycoproteins present. In addition, two-phase polyethylene glycol-dextran separation of disrupted Pichinde virus (Vezza et al., 1977) and CsCl centrifugation of detergent-disrupted Pichinde, Tacaribe and Tamiami viruses (Gard et al., 1977), showed that the viral glycoproteins were found only in the fractions containing envelope fragments. Among the viruses containing both glycoproteins, G1 appears to be more glycosylated than G2 (Martinez Segovia and De Metri, 1977; Vezza et al., 1977; Buchmeier et al., 1978). Since G2 is more easily removed from the virus than G1 by treatment with Nonidet P-40 in low salt conditions (Ramos et al., 1972), one may expect that G1 is more tightly bound within the viral envelope. Pichinde virus contains about 440 and 390 molecules of G2 and G1 respectively per virion (Vezza et al., 1977). The G2 and G1 proteins represent 11-13.8% and 10.4-16.5% of the Pichinde viral proteins, as determined by protein labelling (Buchmeier et al., 1977). No significant amount of sulfate or phosphate was detected in the structural polypeptides of Pichinde, Tacaribe and Tamiami viruses (Vezza et al., 1977; Gard et al., 1977).

A high-molecular-weight polypeptide has been detected in minor amounts, in Pichinde virus and in infected cells by immunoprecipitation (Harnish et al., 1981). This L protein, of approximately 200,000 MW, was not glycosylated and did not chase into smaller cleavage products. During a series of pulse-chase experiments, a 79,000MW glycoprotein, GPC, was detected in infected cells and was subsequently cleaved into GP1 (52,000MW) and GP2 (36,000MW). The L, NP and GPC were found to be unique by two-dimensional tryptic peptide mapping. The NP, GP1 and GP2 of LCM virus were also found to be distinct by tryptic peptide mapping (Buchmeier et al., 1978). By deduction, the L protein may represent the RNA-dependent RNA polymerase that is known to be associated with the virion (Harnish et al., 1981).

The nature of the remaining minor proteins detected in purified arenaviruses is unclear. The different observations are probably reflections of variations in the time of labelling of viral proteins, in the time of virus harvest and in the purification procedures used. The P proteins whose molecular weights range from 75,000 to 79,000, may represent the glycoprotein precursor, GPC. Another possibility is that these proteins are not virus-specific. The 77,000MW P protein described by Vezza et al. (1977) was also observed in the virus preparations of Harnish et al. (1981) however, this polypeptide did not react with hamster immune serum and was also evident in mock-infected cells.

Some of the minor smaller proteins seen in purified virus may represent cleavage or breakdown products of the virus-specific polypeptides. Support of this idea comes from the results of Harnish et al. (1981). Six small proteins ranging in molecular weights from 14,000 to 48,000 were detected in infected cells by immunoprecipitation. All of these proteins were shown to be related to the NP by tryptic peptide mapping. Whether the small peptides are due to proteolysis or to premature termination of NP translation is currently not known.

In summary, arenaviruses appear to contain two or three major proteins and one minor protein. All arenaviruses thus far examined, contain a major nucleocapsid polypeptide, NP and one or two major glycoprotein species. If the virus contains two glycoproteins i.e. GP1 and GP2, then it appears that these two proteins are synthesized from a common precursor, GPC. The L protein appears in trace amounts in purified virus and may represent the RNA-dependent RNA polymerase. The nature of the remaining minor proteins is unclear.

E. Virus-associated enzymes

Carter *et al.* (1974) showed that an RNA-dependent RNA polymerase activity was associated with purified Pichinde virus preparations. The enzymatic activity was detected after disruption of virus by nonionic detergent and required both Mg^{2+} and Mn^{2+} for maximal activity. The *in vitro* polymerization product formed an RNase-resistant product when hybridized with viral RNA. Further analysis of the enzymatic activity of Pichinde virus resulted in the detection of three types of polymerase activity; an RNA transcriptase activity, a poly(A) polymerase activity and a poly(U) polymerase activity (Leung, 1978; Leung *et al.*, 1979). The transcriptase activity was associated with the RNP complex while the poly(A) and poly(U) polymerase activities were found associated with the ribosomes. The transcriptase activity, *in vitro*, resulted in the synthesis of long strands of heteropolymeric RNA that hybridized to viral RNA but not to ribosomal or transfer RNA. The L protein, described by Harnish *et al.* (1981), is a likely candidate for the RNA transcriptase. The L protein is virus-specific. It is present in purified virus in small amounts. The L protein is synthesized in relatively large amounts in Pichinde virus-infected BHK cells and appears to rapidly associate with an RNP or RNP-like complex shortly after synthesis (A. Ramsingh, unpublished observations).

The ribosome-associated polymerases exhibited properties similar to those of poly(A) and poly(U) polymerases present in the polyribosome fractions prepared from uninfected BHK-21 cells. The poly(A) polymerase could accept, as a primer, exogenous viral complementary RNA which had been synthesized by the viral transcriptase in vitro. This observation raises the possibility that the poly(A) polymerase may function in vivo to polyadenylate messenger RNAs synthesized by the virion-associated RNA transcriptase. Although the messenger RNAs of arenaviruses have not been rigorously characterized, it seems likely that the mRNAs of Pichinde virus are polyadenylated (Leung et al., 1977; A. Ramsingh, unpublished observations). No comparable role for the poly(U) polymerase could be postulated. Alternatively, the poly(A) and poly(U) polymerases, present in purified virus, may represent adventitious incorporation along with host-cell ribosomes and thus, may have no role in viral replication.

IV. Replication of Arenaviruses In Vitro

The replication of arenaviruses is still a poorly understood process. The mechanisms underlying acute and persistent infections need to be determined before we can understand the biological phenomena associated with arenavirus infections. Since arenaviruses cause acute and persistent infections in several mammalian cell lines, most of the information on arenavirus replication is derived from in vitro studies using BHK-21, Vero and L cells.

A. Cytopathogenicity

Arenavirus replication, in vitro, is generally associated with little or no cytopathogenic effects. Cytopathogenicity is correlated with the strain of virus and the cells used for infection (Popescu and Lehmann-Grube, 1976). Both Pichinde and LCM viruses will readily produce cytopathology in MDCK and PK-15 cells (Dutko and Pfau, 1978). There seems to be a correlation between defective interfering (DI) particle production and cytopathogenicity since lack of DI virus production is associated with cytopathology. The host cell can apparently influence DI virus production. This phenomenon is not limited to arenavirus replication, since ortho- and para-myxoviruses likewise do not induce the synthesis of DI virus in certain cells (Choppin and Compans, 1975). Although arenavirus cytopathogenicity, as judged by normal rates of DNA, RNA and protein synthesis in the infected cells, can be minimal, virus infection can lead to some cellular alterations. For example, LCM virus-persistently-infected-murine-neuroblastoma cells have a suppressed production of neurotransmitter (Oldstone et al., 1977). In other arenavirus-infected cells, the histocompatibility antigens may be altered and this may influence the animal's immune response to the infected cells (Oldstone, 1975; Zinkernagel and Doherty, 1977).

B. Growth cycle

Arenaviruses are adsorbed to cells in 60 to 120 minutes (Pfau, 1974). Maximum adsorption of LCM, Pichinde and Machupo viruses occur within 1.5 to 2 hours. Penetration of LCM virus is complete after 45 minutes at 37°C and uncoating of virus occurs within 2 hours. The latent period usually lasts 6 to 8 hours (Lehmann-Grube, 1971; Lehmann-Grube et al., 1975; Buchmeier et al., 1978) but may be shortened to 3 to 4 hours (Pedersen and Volkert, 1966; Dutko and Pfau, 1978). This is followed by a rapid rise in virus production with maximum virus titers detected 24 to 36 hours after infection. The time of maximum virus production is dependent on the multiplicity of infection, temperature and amount of DI particles present in the inoculum (Pedersen, 1979). The maturation process of arenaviruses has been examined mainly by electron microscopy and is, as yet, poorly understood. Progeny virions are released from the plasma membrane by budding. During this time, host-cell ribosomes are incorporated into the virions.

The replicative cycle of Pichinde virus in an acute infection has recently been analyzed (Dimock et al., 1982). Plaque-purified Pichinde virus, prepared under conditions designed to limit the amount of DI virus produced, was used to infect BHK cells at low multiplicities (0.1 pfu/cell). Cells were examined daily for 10 days for virus production, viral polypeptide synthesis and viral genomic RNA synthesis. The results showed that replication of Pichinde virus in BHK cells is tightly controlled and that this controlling mechanism is reflected at the levels of virus release, polypeptide synthesis and accumulation of genomic RNAs. Viral protein synthesis and infectious virus production peaked at 2 and 3 days, respectively, post-infection followed by a steady decline. The kinetics of accumulation of both L and S genomic RNAs paralleled those of infectious virus production and viral protein synthesis. The authors suggest that regulation of Pichinde virus replication early during an acute infection of BHK-21 cells may not be dependent upon the generation of defective interfering virus whose absence was inferred and not directly tested. The relationship between regulation of virus replication and generation of DI virus could have been further investigated by comparable studies in cells that restrict DI virus production, such as MDCK or PK-15 cells. If Pichinde virus replication was similarly regulated in these cells, then the correlation between regulation of virus replication and generation of DI virus would have been better substantiated.

Two new RNA species (MWs approx. 1.0×10^6 and 0.5×10^6) were detected by Dimock et al. (1982) in purified virus that was harvested late in infection. These 2 RNA species were not related to the L or S genomic RNAs as determined by hybridization analysis with cDNA probes. The cDNA probes were synthesized from individual L and S RNA species that had been recovered from agarose gels. A major limitation of this procedure is the presence of contaminating RNAs in the isolated L and S RNA preparations, particularly in the S RNA preparation which may contain degraded L RNA and 28S RNA. This results in the cDNA probe cross-hybridizing with contaminating RNA species as was noted by Dimock et al. (1982). A second limitation of this procedure is the quality of cDNA made when the RNA template has been isolated from agarose gels. The isolated RNA contains agarose components that inhibit transcription by reverse transcriptase. Thus, the cDNA probes are probably not representative of the entire L and S RNAs. The conclusion therefore, that the 2 RNA species, X and Y, are unrelated to the genomic L and S RNAs, is not valid because of the limitations in the procedure used. These 2 RNA species, X and Y, are suspiciously similar to the additional RNA species seen in a late harvest (5 days post infection) of Tacaribe and Tamiami viruses (Vezza et al., 1978) and in LCM virus harvested from cells infected with both standard and DI virus (Dutko, 1981). Many questions concerning the nature of these 2 RNAs X and Y, need to be answered. These questions have already been raised in section IIIC.

Clearly, suitable molecular probes are required to determine the identities of the additional RNA species and to examine the interrelationships among the various RNAs. Such probes could be obtained by constructing cDNA clones for the different RNA species.

C. Viral persistence _in_ _vitro_

Persistently-infected cells _in_ _vitro_ can be readily established with several arenaviruses (Pedersen, 1979). Chronically-infected cells appear morphologically identical to uninfected cells and have similar growth characteristics. However, persistently infected cells resist superinfection with both homotypic and certain heterotypic arenaviruses, yet are capable of supporting heterologous virus growth (Lehmann-Grube, 1971; Welsh _et_ _al_., 1975). The underlying mechanism for the establishment and maintenance of persistently-infected cell cultures is currently unknown. DI viruses may play a role in viral persistence _in_ _vitro_. These defective-interfering particles interfere with the replication of standard virus (Huang, 1973). Furthermore, DI virus production is a common feature of arenavirus infections in cell cultures (Lehmann-Grube _et_ _al_., 1969; Staneck _et_ _al_., 1972; Welsh _et_ _al_., 1972). DI particles of LCM virus appear shortly before maximal synthesis of infectious virus and are detected at highest concentrations after the peak of infectious virus production (Welsh and Pfau, 1972). There is a cyclic variation in infectivity and interference activity and also a variation in the percentage of antigen-positive cells as detected by immunofluorescent staining (Lehmann-Grube _et_ _al_., 1969;

Staneck et al., 1972; Hotchin, 1974; Hotchin et al., 1975; Welsh and Oldstone, 1977). The cyclic period lasts about 20-50 cell passages after which DI activity is detected but little or no infectious virus is measured. Viral antigen can be detected by immunofluorescence, in the cytoplasm of the majority of cells. However, in LCM virus-persistently-infected cells, few cells express viral antigens at the cell surface (Cole et al., 1973; Welsh and Oldstone, 1977). Very little information is available on the biochemical properties of DI particles mainly because of the difficulty of separating DI particles from standard virus by physical means. Welsh and Buchmeier (1979) showed that LCM DI particles, purified from cultures of persistently infected BHK-21 and L929 cells, were slightly less dense than standard virus in sucrose gradients. The protein composition of DI and standard infectious virus were similar. Studies directed at characterizing the RNAs of DI virus have not yielded consistent results. Welsh et al. (1975) reported that the RNAs of LCM virus produced by acutely- and persistently-infected BHK-21 cells showed little difference. Pedersen (1979) later showed that LCM DI particles contained only a single RNA species, a 32S RNA. Dutko et al. (1976) observed that the gel profile of RNA extracted from purified standard Pichinde virus, was resolved into 6 RNA species, i.e. 31, 28, 22, 18, 15 and 4-6S RNAs. However, two types of RNA profiles were observed in Pichinde DI virus preparations. DI virus synthesized up to the 175th cell generation after the initial infection, lacked the 22S and 15S RNAs but also seemed to progressively lose the 31S RNA and acquire a "new" 20S RNA species. Dutko (1981) later showed that the virions from cells infected with only LCM virus contained two viral RNAs (L RNA = 2.85 X 10^6, S RNA = 1.3 X 10^6MW). However, the virions from cells coinfected with standard and DI LCM virus contained 2 additional RNAs (A = 1.1 X 10^6, B = 0.6 X 10^6MW). Although RNase T1 fingerprints showed that the A and B RNAs are unique, the small number of characteristic RNase T1-resistant oligonucleotides did not allow a determination of the origin (L or S RNA) of each of these 2 new species. Analysis of the RNAs of DI Tacaribe virus from persistently-infected BHK cells, revealed that the L and S RNAs of standard virus were absent and instead, 5 new RNA species with MWs of 1.1 X 10^6. 0.9 X 10^6, 0.5 X 10^6, 0.4 X 10^6 and 0.2 X 10^6 were observed (Gimenez and Compans, 1980). Clearly, further studies are necessary to characterize the genetic makeup of arenavirus DI particles. From where do the "new" RNA species originate? Are they related to the L and S RNAs? Suitable molecular probes are necessary to answer these questions. Such probes could be obtained by constructing cDNA clones containing the genetic information (the L and S RNAs) from standard infectious virus and from DI virus particles. Radiolabelled cloned DNAs would serve as excellent probes for determining the interrelationships among the different RNA species by RNA blot analyses. Can some of the "new" RNA species associated with DI virus production, be of cellular origin? If so, what role do these RNAs have?

D. Transcription and replication of viral RNA

Currently, little is known about the transcription of viral RNA in arenavirus-infected cells. The genomic RNA of Pichinde virus is of negative polarity and therefore cannot directly function as mRNA (Leung et al., 1977). In addition, an RNA-dependent RNA polymerase activity is found in Pichinde virus (Carter et al., 1974, Leung et al., 1979). This enzyme is necessary for the in vitro transcription of genomic RNA and a similar role is postulated for its function in vivo.

The mRNAs of arenaviruses have not been well characterized. The observation that mRNAs of Pichinde virus could be retained on oligo [dT] cellulose suggested that the mRNAs of Pichinde virus are polyadenylated (Leung et al., 1977). Preliminary data suggest that the mRNAs of Pichinde virus are polyadenylated, full-length transcripts of the genomic RNAs (A. Ramsingh, unpublished observations).

How does viral RNA replicate? Again, little is known about this process. Inhibitors of DNA synthesis such as 5-bromodeoxyuridine and 5-iododeoxyuridine do not inhibit the replication of arenaviruses (Pfau, 1974). This observation taken together with the absence of a virion-associated RNA-dependent DNA polymerase (Carter et al., 1974), suggest that viral RNA replication is not mediated through a DNA intermediate. However, if viral RNA synthesis is similar to that described for other negative-strand RNA viruses, one would expect synthesis of full-length antigenomic RNA which would serve as a template for synthesis of genomic RNA. Genomic and antigenomic RNAs of negative-strand RNA viruses are not found free in infected cells but are tightly associated with ribonucleoprotein structures (Lazzarini et al., 1981). Protein synthesis seems to be required for the synthesis of antigenomic and genomic RNAs. This requirement may be traced to the need for a continuous supply of nucleocapsid proteins that associate with the viral RNAs.

Much work remains to be done in the area of arenavirus replication. The RNA transcriptase needs to be identified. If the enzymatic activity resides on the L protein, then this protein should be present in the RNP complexes in arenavirus-infected cells. The possible role of exogenous primers in the transcription of viral RNAs needs to be examined. Since there are at least 3 primary gene products in arenavirus-infected cells and only 2 viral genomic RNAs, then one RNA species must encode at least 2 products. The mRNAs should be characterized as to whether they are polycistronic or monocistronic and whether they are polyadenylated or not. The kinetics of mRNA and viral RNA syntheses also need investigating. The type of control mechanism that operates in the regulation of virus replication and in persistent infection needs to be examined. Control may be exerted at the transcriptional level or at the gene level.

In the latter case, this may result in amplification of specific gene(s). These are only some of the areas that need to be addressed before we can better understand the events in the transcription and replication of the viral genome. Further work also needs to be done in the areas of virus assembly and maturation.

E. Viral antigens

The antigens of arenaviruses can be broadly defined as being type-specific or cross-reacting. Type-specific antigens are usually detected in neutralization tests while cross-reacting antigens are detected in complement-fixation assays and immunofluorescence tests. More recently, monoclonal antibodies are being used to define more precisely the antigenic relationships among the arenaviruses (Buchmeier and Oldstone, 1981).

Smadel _et_ _al_. (1939) showed that LCM virus-infected guinea pig tissues contained an antigen with complement fixation (CF) activity. This antigen was "soluble" and could also be separated from LCM virus. A similar antigen was found in Junin virus-infected animals (Coto, 1974) and in Pichinde virus-infected cells (Buchmeier _et_ _al_., 1977). CF antigenic activity was detected in disrupted LCM and Pichinde virus preparations (Gschwender _et_ _al_., 1976; Rawls and Buchmeier, 1975). Initially, the CF antigen was thought to consist of two antigens: a thermolabile antigen and a protease-resistant thermostable antigen (Bro-Jørgensen, 1971). This appears not to be the case since antiserum prepared against the RNP complex of Pichinde virus reacted with the heat-resistant, soluble antigen (Buchmeier _et al_. 1977) and antiserum prepared against the soluble antigen precipitated the major polypeptide of the viral RNP (Buchmeier and Oldstone, 1978). Thus, the major antigenic determinant of the CF antigen resides in the nucleoprotein. The thermolabile and thermostable antigens are probably the result of proteolysis of the nucleoprotein (Buchmeier _et al_., 1977).

Viral antigens, as detected by immunofluorescence, appear in the cytoplasm and on the surface of infected cells. The major antigen detected intracellularly is represented in the virion core, presumably the NP, while the antigens detectable at the cell surface are thought to be represented at the virion surface, presumably the glycoprotein(s) (Lehmann-Grube _et al_., 1975; Gschwender _et al_., 1976; Buchmeier _et al_., 1977). During acute infection, intracytoplasmic and cell-surface antigens become detectable at about the time of progeny virus production, i.e. 8 hours post infection (Lehmann-Grube _et al_., 1975). Dimock _et al_. (1982) have shown that in Pichinde virus-infected BHK cells, the percentage of immunofluorescent cells increased concomitantly with virus production. More than 90% of the infected cells remained positive for the presence of nucleoprotein even after viral protein synthesis, virus production and the number of cells producing virus decreased. The question arises as to whether the nucleoprotein has a regulatory role in viral gene expression.

F. Viral replication and host functions

Expression of the viral genome appears to be partly influenced by the host cell. The yield of infectious LCM and Pichinde viruses was found to be approximately tenfold greater from exponentially growing BHK-21 cells than from cells in the stationary phase (Pfau et al., 1973; Rawls et al., 1976). Pichinde viral antigens were not synthesized in cells which had been enucleated with cytochalasin B (Banerjee et al., 1976). Nuclear functions were therefore required for the initiation of antigen production. Studies with actinomycin D showed that at concentrations which inhibit cellular RNA synthesis, the production of infectious virus was also inhibited (Buck and Pfau, 1969; Mifune et al., 1971; Stanwick and Kirk, 1971). Actinomycin D did not inhibit viral antigen synthesis but instead, appeared to block a late step in the replicative cycle of Pichinde virus (Rawls et al., 1976). α-Amanitin which also blocks cellular mRNA synthesis, inhibited the synthesis of Pichinde virus. This inhibitory effect was not observed in an α-amanitin-resistant cell line whose genetic lesion is in the cellular DNA-dependent RNA polymerase II. These observations suggest that a cellular function expressed by the RNA polymerase II is necessary for virus replication (Leung, 1978). RNA polymerase II is responsible for mRNA synthesis in cells (Davidson, 1976) so presumably, host cell mRNA(s) are involved in arenavirus replication. Orthomyxovirus replication is also sensitive to α-amanitin (Scholtissek and Klenk, 1975). This inhibition is probably due to prevention of the synthesis of host mRNAs that are necessary as primers for the synthesis of complementary viral RNAs (Krug _et al._, 1978). Therefore, one of the host DNA functions in arenavirus replication could be a production of short-lived mRNAs that function as primers for the synthesis of complementary viral RNAs (Pedersen, 1979). It should be noted that not all negative-stranded RNA viruses require functional cellular DNA for viral replication since the replication of paramyxoviruses (Choppin and Compans, 1975), bunyaviruses (Obijeski and Murphy, 1977) and vesicular stomatitis virus (Wagner, 1975) is not, or only slightly, inhibited by actinomycin D.

V. Replication of Arenaviruses _In Vivo_

Members of the arenavirus group produce persistent infections in rodents primarily in the two major families, _Muridae_ (e.g. mice, rats) and _Cricetidae_ (e.g. voles, gerbils) (Howard and Simpson, 1980). The arenaviruses have highly restricted natural host ranges (Johnson _et al._, 1973) yet they can be used to infect experimentally a variety of mammals. Since these viruses seem to be maintained in nature by persistently-infected animals, the high degree of natural host specificity may represent the evolution of an appropriate virus-host interaction that permits persistence (Rawls _et al._, 1981). The nature of that virus-host interaction has been extensively studied.

Development of persistence is dependent on several factors such as age at infection and the strain, dose and route of inoculation of the virus (Rawls et al., 1981). Viral persistence is the usual outcome of congenitally acquired virus or infections initiated during the newborn period. These infections are characterized by little or no detectable neutralizing antibodies, lifelong viremia and virus excretion in the urine and saliva. Infections acquired later in life tend to be self-limiting. Infected adult animals tend to clear the viremia and produce large amounts of neutralizing antibodies. The development of viral persistence following congenital or newborn infections but not following infections of adult animals seem to be related to an immature or defective immune system. The immune response to arenavirus infection has been reviewed by Rawls et al (1981). The key observations are the following. The cell-mediated immune response plays a major role in the pathogenesis of arenavirus disease. Fatal LCM virus infections develop only in immunologically mature adults and seems to be a consequence of the damage resulting from the interaction of virus-specific cytotoxic T lymphocytes with LCM virus-infected target cells. Persistently infected animals appear to have a specific unresponsiveness in effector T lymphocyte function. This unresponsiveness may be attributed to T suppressor cells which regulate the generation of T effector cells. Thus, persistent arenavirus infection could be associated with an enhanced T suppressor response. The role of DI virus in persistently-infected animals has also been examined and the results are not conclusive.

Certain arenaviruses cause severe hemorrhagic disease in man i.e. Lassa, Junin and Machupo viruses. The underlying mechanisms by which these viruses cause disease are currently not understood. There is no evidence that either immunopathological or allergenic processes play a role in causing disease and it seems more likely that direct virus damage to cells is the underlying cause (Howard and Simpson, 1980). Johnson _et_ _al_. (1973) suggested the following pathogenesis following arenavirus infection in man. Viral entry is via the alimentary or upper respiratory tract. Virus is gathered up in local lymphoid tissue or lymph nodes where it first replicates. It then invades the reticuloendothelial system to include those cells involved in the immune and cellular immune responses and thus, impairs the host's defense mechanisms. The virus causes, either directly or indirectly, capillary damage which leads to capillary fragility, hemorrhages and hypovolaemic shock. The malfunction of various organs may be attributed to capillary damage and edema of the parenchyma rather than direct cell damage. As the disease regresses, there is no permanent damage since little cytolysis occurs. However, in severe cases, cytopathic damage may be significant.

Acute hemorrhagic disease due to Machupo and Junin viruses represents serious public health problems in Boliva and Argentina respectively. More recently, Lassa virus has attained public notoriety owing to its association with severe, febrile illness among missionaries and travellers returning from rural parts of West Africa. Before we can effectively treat these diseases, a great deal of further work is necessary to unravel the secrets of the biology of arenaviruses.

CHAPTER 2

MATERIALS AND METHODS

I. Tissue Culture

Two cell lines were used in these experiments i.e. BHK-21 clone 13 cells (obtained from C. Howard, London School of Hygiene and Tropical Medicine, England) and Vero cells. They were grown in Dulbecco-modified Eagle's medium supplemented with 5% heat-inactivated fetal calf serum. Sodium penicillin G and streptomycin sulphate (Gibco Canada, Burlington, Ontario) was routinely added to the medium to control bacterial contamination. The pH of the medium was adjusted to approximately 7 by the addition of 7.5% sodium bicarbonate.

BHK cells were used for growing both Pichinde virus and vesicular stomatitis virus. These cells were grown in 150cm^2 plastic flasks or 490cm^2 plastic roller bottles (Corning Glass Works, Corning, N.Y.). Confluent cell monolayers in 150cm^2 flasks were trypsinized as follows. After decanting the tissue culture medium, the cells were incubated with 4ml 10 X trypsin-EDTA (Gibco Canada, Burlington, Ont.) at room temperature for 2-5 min until the cells detached from the plastic surface. Confluent cell monolayers were subcultured at a ratio of 1:5 to 1:10. Periodically, cell suspensions were frozen in culture medium containing 10% glycerol or 10% dimethylsulphoxide (DMSO) and stored in liquid nitrogen.

Vero cells were used for titrating Pichinde virus by plaque assay and were grown in 60mm Petri dishes. Trypsinization was carried out as previously described.

II. Pichinde Virus

A. Preparation of stock virus

90% confluent monolayers of BHK cells in 150cm^2 flasks were infected with Pichinde virus at a multiplicity of 0.1-0.2 pfu/cell. After adsorption at 37°C for 1 hr, the unadsorbed virus suspension was replaced with 25ml of tissue culture medium which was changed after 24 hr. Extracellular fluid was harvested 48 hr post infection. After a clarifying spin at 8,000g for 15 min at 4°C, the supernatant was dispensed into 1ml and 2ml aliquots and stored at -80°C.

B. Plaque assay

Confluent Vero cell monolayers in 60mm Petri dishes were inoculated with serial dilutions of virus (10^{-5} to 10^{-8}). After a 1 hr adsorption at 37°C, 4.0ml of overlay medium was added. The overlay medium consisted of a 1:1 mixture of 2X overlay (Eagle's or Hank's MEM 2X supplemented with 20% fetal calf serum, glutamine, penicillin/streptomycin and mycostatin and buffered with 30mM Hepes buffer, pH7.0) and 2% agar. After solidification of the overlay medium at room temperature, the plates were incubated at 37°C for 3-4 days. A second overlay prepared as described above but also containing 0.04% neutral red, was added and the plates were incubated for an additional 1-2 days at 37°C. Clear plaques were counted and the virus titer was calculated.

C. Purification of virus and extraction of viral RNA

90% confluent monolayers of BHK cells in 150cm^2 or 490cm^2 flasks were infected with Pichinde virus at a multiplicity of 0.1-1.0 pfu/cell. After a 1 hr adsorption at 37°C, the unadsorbed virus suspension was replaced with fresh medium. The medium was changed after 24 hr and extracellular fluid was collected at 24-48, 48-72 and 72-96 hours post-infection. After a clarifying spin at 8,000g for 15 min at 4°C, the supernatant was adjusted to 0.5M NaCl and 7% polyethylene glycol (average M.W. 6,000-7,500). After mixing at 4°C for 3 hr, the suspension was pelleted at 8,000g for 15 min at 4°C. The pellets were resuspended in TNE buffer (0.01M Tris-HCl, pH7.5, 0.1M NaCl, 0.001M EDTA). This suspension may then be stored at -80°C. After treating with 3mg/ml Pronase (Sigma Chemical Co., St. Louis, Mo.) at 37°C for 30 min to remove contaminating cellular debris, the suspension was layered onto a 10% (4ml) and 40% (3ml) (w/v) discontinuous Renografin (E.R. Squibb and Sons Ltd., Montreal, Que.) gradient in TNE buffer. After centrifugation at 150,000g for 1 hr at 4°C in an IEC SB-283 rotor the visible virus band, at the interface of the 10% and 40% solutions, was collected. It was diluted at least 1:1 with TNE buffer and layered onto a continuous, linear Renografin gradient (10-40%, w/v). After centrifugation at 150,000g for 2 hr at 4°C, the visible virus band was collected and diluted 1:1 with TNE buffer. Virions were disrupted in 0.1% β-2-mercaptoethanol and 0.1% SDS. The suspension was extracted with an equal volume of a 1:1 (v/v) mixture of 90% phenol (saturated with TNE) and chloroform. The organic phase was re-extracted with TNE buffer. The pooled aqueous phase was mixed with 2.5 volumes of 95% ethanol and RNA was precipitated at -20°C overnight. RNA was pelleted at 16,000g for 30 min at 4°C and resuspended in autoclaved water ; the RNA concentration was determined by reading the absorbance at 260nm in a Beckman A25 spectrophotometer. RNA was routinely stored in 70% ethanol and 125mM sodium acetate at -20°C. Prior to use, RNA was pelleted in an Eppendorf centrifuge for 10 min at 4°C and air dried. A 24-48 hr harvest of virus from approximately 3 X 10^9 cells grown in 30 roller bottles yielded 50-60μg of RNA.

D. Preparation of labelled viral RNA

90% confluent monolayers of BHK cells in 490cm^2 roller bottles were infected with Pichinde virus at a multiplicity of 0.1-0.2 pfu/cell. After a 1 hr adsorption at 37°C, the inoculum was replaced with 25ml of tissue culture medium containing 1μCi/ml [^{3}H]-uridine (specific activity 27.6 Ci/mmol; New England Nuclear Canada, Dorval, Que.). The medium was changed after 24 hr and fresh medium containing 1μCi/ml [^{3}H]-uridine was added. Extracellular fluid was harvested 48 hr postinfection and processed as described in section II-C.

III. Vesicular Stomatitis Virus

A. Purification of virus and extraction of viral RNA

Confluent monolayers of BHK cells in $150cm^2$ flasks were infected with VSV (Indiana serotype) at a multiplicity of 0.5 pfu/cell. After a 1 hr adsorption at 37°C, fresh medium was added. Extracellular fluid was harvested 24 hr post infection and clarified at 8,000 g for 15 min at 4°C. The supernatant was adjusted to 0.5M NaCl and 7% polyethylene glycol. After mixing at 4°C for 3 hr, the suspension was pelleted at 8,000g for 15 min at 4°C. The pellet was resuspended in TNE buffer and layered onto a continuous, linear sucrose gradient (10-30%, w/v). After centrifugation at 80,000g for 1 hr at 4°C, the visible virus band was collected and diluted 1:1 with TNE buffer. Virions were disrupted and RNA extracted as described for Pichinde virus (section II-C).

B. Preparation of labelled viral RNA

After infecting BHK cells with VSV as described in section III-A, the inoculum was replaced with 20ml of fresh medium containing 0.5μCi/ml[^{3}H]-uridine (specific activity 27.6 Ci/mmol, New England Nuclear Canada, Dorval, Que.). Extracellular fluid was harvested 24 hr post infection and processed as described in section III-A.

IV. Cellular Ribosomal RNA

A. Extraction of cellular ribosomal RNA

Confluent monolayers of BHK cells in 150cm^2 flasks were washed with cold phosphate-buffered saline (PBS) (0.14M sodium chloride, 3mM potassium chloride, 8mM disodium hydrogen phosphate, 1mM potassium dihydrogen phosphate). Cells were harvested by scraping with a rubber policeman followed by centrifugation at 3,000g for 10 min at 4°C. The cell pellet was resuspended in NENSH buffer (100mM NaCl, 10mM EDTA, 50mM sodium acetate, pH5.1, 0.5% SDS and 50U/ml sodium heparin) containing 250μg/ml proteinase K and incubated at 37°C for 30 min with intermittent vortexing. After extraction with a 1:1 mixture of phenol and chloroform, the aqueous phase was collected. The phenol phase was reextracted with NENSH buffer. The aqueous phases were pooled and back extracted with a 1:1 mixture of phenol and chloroform. The aqueous phase was collected and nucleic acids were precipitated with 2.5 volumes ethanol at -20°C overnight. After centrifugation at 16,000g for 30 min at 4°C, the pellet was digested with 10μg/ml DNase at 4°C for 30 min to remove contaminating DNA. This was followed by digestion with 100μg/ml proteinase K in 0.5% SDS at room temperature for 20 min to remove contaminating protein. After extraction with a 1:1 mixture of phenol and chloroform, the aqueous phase was collected. The remaining phenol phase was re-extracted with TNE and the aqueous phases were pooled. After precipitating with 2.5 volumes of ethanol at -20°C, the suspension was centrifuged at 16,000g for 30 min at 4°C and the pellet resuspended in TE buffer (0.01M Tris-HCl pH7.5, 0.001M EDTA) and 1.5M lithium chloride and incubated at 4°C overnight. Since DNA, tRNA and heparin are soluble in lithium chloride whereas ribosomal RNA (rRNA) is not, rRNA was pelleted by centrifugation at 16,000g for 30 min at 4°C. The RNA pellet was resuspended in autoclaved water and the optical density of a small aliquot was determined at an absorbance of 260nm to determine the RNA concentration. RNA was stored frozen or in 70% ethanol and 125mM sodium acetate at -20°C.

B. Preparation of labelled cellular ribosomal RNA

Confluent monolayers of BHK cells in 150cm^2 flasks were washed 5X in phosphate-free medium. 10ml of phosphate-free medium containing 25μCi/ml[^{32}P]-phosphate (New England Nuclear Canada, Dorval, Que.) was added to each flask followed by an overnight incubation at 37°C. RNA was extracted as described in section IV-A.

V. Estimations of the Molecular Weights of Pichinde Viral RNAs by Gel Electrophoresis under Different Denaturing Conditions

A. Agarose gel electrophoresis in 10mM methylmercury hydroxide

Agarose gel electrophoresis in 10mM methylmercury hydroxide was modified according to the method of Lehrach et al. (1977). Four different concentrations (0.75, 1.0, 1.25, 1.5%) of agarose were prepared in electrophoresis buffer (50mM boric acid, 5mM sodium borate, 10mM sodium sulfate, 100μM EDTA, pH 8.1) and kept at 56°C until ready for use. Methylmercury hydroxide (Alpha Products, Boston, Mass.) was added to a final concentration of 10mM and the mixtures were poured into siliconized, cylindrical 20 X 0.7cm gel tubes. The top of the gel was sliced off prior to sample application.

The RNA sample (4μg/gel) in 25mM boric acid, 2.5mM sodium borate, 5mM sodium sulfate, 50μM EDTA, pH 8.1, 10% glycerol was denatured by the addition of methylmercury hydroxide to 10mM. After a 15 min incubation on ice, bromophenol blue was added to 0.004%. Electrophoresis buffer was recirculated between the lower and upper reservoirs. Electrophoresis was carried out at 7mA per tube at room temperature for 4 hr inside a fume hood. Gels were stained in the dark in 2μg/ml ethidium bromide, 0.5M ammonium acetate. Gels were then photographed under shortwave UV light with a Polaroid camera and high-speed film Land type 107 using a red filter. In some instances, gels were cut into 2-mm slices with a Bio-Rad gel slicer and processed for scintillation counting. After alkali digestion of RNA at 37°C, an aqueous counting scintillation cocktail was added and the radioactivity was measured in a Beckman scintillation counter.

All operations involving methylmercury hydroxide were carried out in a fume hood with the operator wearing disposable gloves. Contaminated disposable material was discarded with radioactive waste. Non-disposable items were rinsed in a 1% solution of β-2-mercaptoethanol and then cleaned.

B. Electrophoresis of RNA in composite polyacrylamide-agarose gels after denaturation in glyoxal and DMSO

Electrophoresis was carried out as described by McMaster and Carmichael (1977) with some modifications. Four different concentrations of acrylamide (1.0, 1.2, 1.4 and 1.6%) were prepared in 0.5% agarose, 10mM sodium phosphate, pH 7.0, 0.1% ammonium persulfate, 0.001% TEMED and poured into siliconized, cylindrical 20 X 0.7cm gel tubes. The top of the gel was sliced off prior to sample application.

Prior to use, 1ml aliquots of glyoxal and DMSO were deionized by repeated passage thrice through a 3ml syringe filled to the 2ml mark with autoclaved, mixed-bed ion-exchange resin, AG-501-X8 (Bio Rad Laboratories). The RNA preparations (4μg/gel) in 0.25% SDS, 0.067% EDTA, 10mM phosphate buffer, pH 7.0, 15% glyoxal, 50% DMSO were heat-denatured at 56°C for 2 min followed by a 15 min incubation at room temperature. Bromophenol blue was added to 0.05%. Electrophoresis buffer (10mM sodium phosphate buffer, pH 7.0) was recirculated between the lower and upper reservoirs. Electrophoresis was carried out at 1mA/tube overnight at room temperature. Gels were stained in 3.0μg/ml acridine orange in 10mM phosphate buffer, pH 7.0 for 30 min at room temperature and destained in the dark in 10mM phosphate buffer, pH 7.0 for 3.5 hr at room temperature. The RNA bands in the gels were photographed as described in section V-A.

C. Agarose gel electrophoresis in 2.2M formaldehyde

Agarose gel electrophoresis in 2.2M formaldehyde was modified according to Lehrach _et_ _al_. (1977). A 1% agarose suspension was prepared in 2.2M formaldehyde in 0.018M Na_2HPO_4-0.002M NaH_2PO_4 and poured into siliconized, cylindrical gel tubes.

The top of each gel was sliced off prior to sample application. RNA samples were heated in 2.2M formaldehyde, 50% deionized formamide, 0.018M Na_2HPO_4-0.002M NaH_2PO_4 for 5 min at 60°C after which 20% of a 1:1 glycerol/deionized formamide mixture containing 0.004% bromophenol blue was added. Electrophoresis buffer (2.2M formaldehyde, 0.018M Na_2HPO_4, 0.002M NaH_2PO_4) was recirculated between the upper and lower reservoirs. Electrophoresis was carried out at 2mA per tube at room temperature. Gels were stained in 2μg/ml ethidium bromide in 0.5M ammonium acetate. Gels were photographed as described in section V-A.

VI. Preparation of Primer from Calf Thymus DNA

100mg of calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) in 10ml of 20mM Tris-hydrochloride (pH7.4) and 10mM $MgCl_2$ was digested with 30μg of DNaseI (Boehringer-Mannheim, Dorval, Que., Canada) per ml at 37°C for 30 min. The digestion was stopped by the addition of EDTA to 10mM. The DNA was then denatured by the addition of NaOH to 0.1M followed by heating at 70°C for 10 min. After neutralization with 1N HCl to pH7.0, the DNA solution was deproteinized with water-saturated phenol. Solid urea (Bio-Rad Laboratories, Mississauga, Ont. Canada) and concentrated ammonium carbonate were added to the DNA solution to give final concentrations of 7M and 0.01M (pH8.6) respectively. The DNA sample was applied to a Sephadex G-50 column (72cm X 2.5cm) which had been equilibrated with 7M urea-0.01M ammonium carbonate, pH8.6. The urea-ammonium carbonate buffer had been passed through a DEAE-Sephadex A25 column to remove UV-absorbing materials. The DNA was eluted with the urea-ammonium carbonate buffer at a flow rate of 60ml per hour at room temperature. 5ml fractions were collected. The absorbance of each fraction at 260nm (A_{260}) was determined in a Beckman A25 spectrophotometer. The oligodeoxynucleotide fractions were pooled and desalted by passage through a Sephadex G-25 column (40cm X 2.5cm) equilibrated with sterile water. Elution with sterile water was carried out at room temperature. The oligodeoxynucleotides in the excluded volumes were concentrated by lyophilization and stored at -20°C.

VII. Determining the Optimum Conditions for the *In Vitro* Synthesis of Pichinde cDNA Probes

In order to optimize the conditions for *in vitro* cDNA synthesis, the effects of various concentrations of the components of the reaction mixture were examined. cDNA synthesis was carried out in a final volume of 100μl. The reaction mixture consisted of the following:

a. Tris-hydrochloride pH8.3 : 50mM
b. $MgCl_2$: 8mM
c. dATP : 1mM
d. dGTP : 1mM
e. dTTP : 1mM
f. cold dCTP : 0.0005-0.5mM
g. $[^3H]$dCTP : 5μCi
h. KCl : 0-300mM

i. dithiothreitol (DTT) : 0-10mM

j. bovine serum albumin (BSA) : 0-1000μg/ml

The BSA was purified by chromatography on UMP-agarose (Miles Laboratories Inc., Elkart, Ind.) to remove possible RNase activity.

k. actinomycin D : 0-400μg/ml

l. Pichinde vRNA : 0.01-40μg/ml

m. oligodeoxynucleotide primer : viral RNA template:60:1

n. reverse transcriptase : 0-400 units/ml

The reverse transcriptase obtained from avain myeloblastosis virus was obtained from Joseph Beard, Life Science Inc., St. Petersberg, Fla.

The reaction mixture was assembled on ice and prior to the addition of the reverse transcriptase, the template RNA was heat-denatured at 70°C for 1 min followed by immediate chilling in wet ice. After the addition of the reverse transcriptase, the reaction mixture was incubated at 37°C for 2 hr. The incorporation of [^{3}H]dCTP into acid precipitable material was monitored during the 2 hr incubation period as an indication of the extent of cDNA synthesis.

For cDNA isolation, the viral template RNA was alkali digested. NaOH was added to 0.33N and the reaction mixture was incubated overnight at 37°C in a sealed tube. The reaction mixture was then neutralized by the addition of HCl and desalted by passage through a Sephadex G-50 column equilibrated with TNE and 0.1% SDS. The cDNA fractions in the excluded volume, as monitored by radioactivity, were pooled and the cDNA recovered by ethanol precipitation.

VIII. Isolation of Pichinde Virus L and S RNAs

Pichinde virus L and S RNAs were isolated according to the procedure described by Leung et al. (1981). Pichinde virus was purified as described in section II-C. Virus was then pelleted by centrifugation at 100,000g for 1 hr at 4°C. The virus pellet was suspended in TNE and 0.5% SDS and extracted repeatedly with equal volumes of TNE-saturated phenol/chloroform (1:1). The aqueous phases were pooled and extracted with ether to remove residual amounts of phenol. RNA was precipitated with 2 volumes of 95% ethanol at -20°C overnight. Viral RNA was collected by centrifugation and the RNA pellet was dried under vacuum. The RNA was denatured with 10mM methylmercury hydroxide in electrophoresis buffer (0.05M boric acid - 0.005M $Na_2B_4O_7$-0.01M Na_2SO_4-0.1mM EDTA, pH8.1). After electrophoresis in a 1% agarose gel supplemented with 10mM methylmercury hydroxide, the gel was stained with 5μg of ethidium bromide per ml, 20mM 2-mercaptoethanol and 0.5M ammonium acetate for 30 min. The RNA bands were visualized with short-wavelength UV light and the L and S RNAs were excised with a razor blade. The gel slices were packed into columns of 5ml plastic pipettes with dialysis bags attached to their tips. RNA was electroeluted into the dialysis bags by electrophoresis overnight. The flow of current was reversed for

2 min prior to the termination of electroelution to minimize adherence of RNA to the dialysis membranes. The RNA was extracted repeatedly with n-butanol to remove ethidium bromide. RNA was then precipitated with 1/10th volume of 4M LiCl and 2 volumes of ethanol at -20° overnight. The L or S RNA was collected by centrifugation, washed once in 70% ethanol, 0.1M NaOAc and stored in 70% ethanol until use.

IX. Hybridization of $[^3H]$cDNA to RNA

The procedure for hybridization of $[^3H]$cDNA to RNA was described by Leung _et_ _al_. (1981). The hybridization mixture contained 0.3M NaCl, 0.01M Tris-HCl (pH7.5), 0.05μg of $[^3H]$cDNA, 1μg viral RNA and 50μg/ml carrier yeast tRNA. Hybridization was carried out at 70°C for 1 to 22 hr. The degree of annealing was monitored as the percentage of radioactivity resistant to S1 nuclease digestion in the presence of 0.05M Tris-acetate (pH4.4) and 1mM $ZnSO_4$. Results were expressed as Rot [Rot=(the concentration of RNA in moles per liter) X (times in seconds)].

X. Preparation of Antiserum to Pichinde Viral Antigens

Five week old male LVG hamsters (Charles River) were each injected intraperitoneally with 2 X 10^3 pfu of virus stock in a total volume of 0.2ml. They were boosted 6 weeks later with the same dose of virus, again by intraperitoneal inoculation. Two weeks after boosting, the hamsters were bled by cardiac puncture. The blood was allowed to clot at 4° overnight. Serum was collected after low speed centrifugation, aliquoted and stored at -50°C.

XI. Preparation of a Synthetic Peptide Coupled to Bovine Serum Albumin

The work described in this section was part of a collaborative effort with A. Taneja and R. Hodges (Department of Biochemistry, University of Alberta).

Abbreviation for chemicals:

Boc, N^{α}-tert-butyloxycarbonyl; DCC, N, N'-dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid, AB-OSu, N-hydroxysuccinimide ester of 4-azidobenzoic acid.

All solvents, resins and reagents used in peptide synthesis have been previously described (Worobec _et_ _al_., 1983). The synthesis of BOC-[1-^{14}C]Gly (Worobec _et_ _al_., 1983) and AB-OSu (Chong and Hodges, 1981) have also been reported. Routine methodologies used in the synthesis of peptides i.e. thin layer chromatography, amino acid analyses and high voltage paper electrophoresis, were carried out as previously described (Worobec _et_ _al_., 1983).

A. Chemical synthesis of a Pichinde peptide [(Gly)^9-P(1-9) amide]

The 9-residue peptide was synthesized by the general procedures for solid-phase peptide synthesis on a Beckman peptide synthesizer (model 990) (Erickson and Merrifield, 1976). Coupling of radioactive Boc-[1-^{14}C]-Gly to the benzhydrylamine resin (0.30 mmol/g resin) resulted in a substitution of 0.28mmol of Boc-Gly per gram of resin as determined by picrate monitoring (Hodges and Merrifield, 1975a). The remaining free amino groups on the resin were terminated by washing with an acetylating mixture (pyridineacetic anhydride-benzene, 3:1:1 by volume, 12.5 ml per gram of resin) for 5 min, followed by acetylation with the same mixture for 60 min. The picrate monitoring established complete acetylation of all the available amino groups.

All amino acids were protected at the α-amino position with Boc groups, and the following side chain protecting groups were used: for Arg, 4-tolylsulfonyl (Tos), for Ser, benzyl (Bzl), for Asn, 4,4'-dimethoxybenzhydryl (Mbh) and for Asp, o-benzyl (OBzl). The Boc groups were removed at each cycle of the synthesis by treatment for 30 min with 25ml of 50% trifluoracetic acid - CH_2Cl_2 (vol/vol). After each deprotection step, neutralization was carried out with 25ml of 5% DIEA-CH_2Cl_2 (vol/vol). Boc-aminoacids (0.435mmol; 3 equivalents) in 4ml of CH_2Cl_2 were added to the peptide-resin (0.5g) followed by a 3.2ml solution of DCC (0.478 mmol) in CH_2Cl_2. Boc-Arg(Tos) was dissolved in 0.3ml of DMF and diluted to 4ml with CH_2Cl_2. Double couplings of 90 min each were performed at each step of the synthesis. The program used for attachment of each amino acid and the picrate monitoring procedure were as previously described (Hodges _et_ _al_., 1981).

The cleavage of the peptide from its resin support was accomplished with hydrofluoric acid at 0°C for 45 min with 10% anisole (vol/vol) as a cation scavenger (Hodges and Merrifield, 1975b). The peptide was extracted from the resin with trifluoroacetic acid. The TFA was evaporated and the residue dissolved in 5% acetic acid and freeze-dried.

B. Purification of synthetic peptide

The crude peptide (150mg) was purified by reverse-phase high pressure liquid chromatography (HPLC) on a Whatman Partisil M9 ODS-2 Column (10mm X 500mm) as follows: Solvent A is 0.1% TFA-H_2O; Solvent B is 0.1% TFA-acetonitrile. The column was equilibrated with an AB mixture (85%A-15%B), and eluted with an increasing linear gradient of B (0.2%/min) for 50 min at a flow rate of 2ml/min. The peptide was detected at 220nm and eluted between 22-25 min. Single injections containing as much as 20mg of crude peptide were injected. The purity of the peptide was verified by high voltage paper electrophoresis at pH6.5 and pH1.8. Amino acid analysis: Asp(1.97), Ser(1.51), Pro(1.02), Gly(1.00), Ile(0.96), Phe(1.00), Arg(1.06).

C. Preparation of covalently linked peptide-BSA complex

The pure C^{14}-labelled peptide, (Gly^9)-P(1-9) amide (2.0mg, 2μmol) was dissolved in aqueous $NaHCO_3$ (1.15mg in 200μl H_2O). To this solution, AB-OSu, (3.45mg, 13.3μmol) dissolved in 200μl of dioxane, was added dropwise over a period of 10 min with consistent stirring. The reaction was allowed to proceed for 1 hr at 0°C and then at room temperature for 24 hr. The reaction of the N-terminal amino group of the peptide with AB-OSu was shown to go to completion by the changed electrophorectic mobility at pH6.5, concomitant with the disappearance of the ninhydrin and radioactive-positive spot and the appearance of the ninhydrin-negative, radioactive-positive spot in the neutral zone. The modified peptide was purified on Sephadex G-25 as previously described (Worobec et al., 1983). The radioactive peptide fractions were pooled and combined with BSA(5.2mg, 76nmol) and lyophilized.

The freeze-dried photoaffinity-labelled peptide -BSA mixture was dissolved in 100µl of 1mM HCl, which had been previously degassed and saturated with nitrogen. This solution was photolyzed for 2 hr in the cold room (4°C), using an RPR 208 preparative reactor (Royonet, The Southern New England Ultraviolet Co., Middletown, Conn.) equipped with 3,500-A° lamps. The reaction mixture was applied to a Sephadex G-50 column as previously described (Worobec et al., 1983). The covalent peptide-BSA complex was pooled and lyophilized. Radioactivity measurements indicated a peptide-BSA ratio of 0.8:1.

XII. Preparation of Antiserum to a Synthetic Peptide Coupled to Bovine Serum Albumin

An 8 amino acid peptide, encoded in the 3' proximal end of the S RNA from nucleotide 87 to 110 inclusive, was synthesized and coupled to a carrier protein, BSA. This coupled peptide was used to raise antiserum in rabbits. Rabbits were bled from the marginal ear vein prior to immunization and serum was collected for use as preimmune sera. Four week old male Flemish rabbits were each injected subcutaneously with 2 X 0.2ml (total of 600µg) of coupled peptide in complete Freund's adjuvant (1:1) on day 0. On day 17, the rabbits were boosted with 0.4ml (600µg) of antigen in incomplete Freund's adjuvant (1:1) at intramuscular sites. A similar boost was given on day 24. Rabbits were bled from the marginal ear vein on day 32. Serum was clarified as previously described. Aliquots of serum were stored at -50°C.

XIII. Radioactive Labelling of Intracellular Proteins in Pichinde Virus-Infected Cells

Subconfluent BHK cell monolayers were infected with Pichinde virus at a multiplicity of infection of 10pfu/cell. Twenty-four hours post infection, the cell monolayer was washed twice with PBS and labelled with 25μCi of L-[^{35}S] methionine (1140Ci/mmol, Amersham Corp.) per ml for 3 hr in 5ml of methionine-free Eagles MEM (Flow Laboratories, Rockville, Md.) supplemented with 5% dialyzed fetal calf serum, 0.15% $NaHCO_3$, glutamine, penicillin and streptomycin per 150cm^2 flask.

XIV. Immunoprecipitation and Gel Electrophoresis of Viral Polypeptides

BHK cells growing in 150cm^2 flasks were harvested by scraping with a rubber policeman, washed 3X with PBS and lysed in 1ml of RIPA buffer (50mM Tris HCl, pH7.2, 150mM NaCl, 0.1% (wt/vol) SDS, 0.1%(wt/vol) sodium deoxycholate, 1% (vol/vol) Triton X-100 and 1mM phenylmethylsulfonyl fluoride) at room temperature for 10 min. Cellular debris and nuclei were removed by centrifugation of the lysate at 10,000g for 15 min at 4°C. 5μl of the appropriate serum and 100μl of a 1:1 dilution of protein A-Sepharose CL-4B beads (Pharmacia Fine Chemicals, Montreal, Que.) in RIPA buffer were added to 1.0ml of cell extract. After mixing at 4°C for 2 hr, the beads were pelleted and washed 5X with RIPA buffer at 4°C. The beads were resuspended in 125mM Tris HCl, pH6.8, 6% (wt/vol)SDS, 20% glycerol and 10% (vol/vol) β-mercaptoethanol and heated for 3 min at 100°C to elute and denature the immune complexes.

Denaturing polyacrylamide gels were prepared according to the method of Laemmli (1970). Samples were electrophoresed in gradient SDS-polyacrylamide slab gels (7.5 to 15% polyacrylamide) at 35mA for 5 hr in 50mM Tris-380mM glycine (pH8.6) - 0.1%(wt/vol) SDS. Protein molecular weight standards (Pharmacia) were included for each gel. Proteins were stained (Paul et al., 1972) with 0.25%(wt/vol) Coomassie brilliant blue in 50%(vol/vol) methanol and 10% glacial acetic acid(vol/vol) for 30 min at room temperature. Gels were destained in 25% methanol and 10% glacial acetic acid overnight. Radiolabelled polypeptides were visualized by a film (Kodak XAR-5) detection method described by Bonner and Laskey (1974).

XV. Molecular Cloning of the Pichinde Viral Genome

A. Synthesis of double-stranded Pichinde cDNA from RNA extracted from purified virus

Double-stranded Pichinde cDNA was synthesized in a sequential reaction. First strand cDNA copies of Pichinde viral RNA (vRNA) were synthesized in a 400µl reaction mixture containing 50mM Tris-hydrochloride, pH8.3, 8mM $MgCl_2$, 0.5mM dithiothreitol, 1mM each of dATP, dCTP, dGTP and dTTP, 16µg of RNA extracted from purified Pichinde virus preparations, 960μg random oligodeoxynucleotide primer (7-10 nucleotides in length) and 300μg/ml bovine serum albumin (BSA). BSA was previously purified by chromatography on UMP-agarose (Miles Laboratories Inc., Elkart, Ind.) to remove possible contaminating RNase activity. These reaction conditions had been previously optimized to give maximal amount of cDNA synthesis for Pichinde viral RNA (Leung et al., 1981). The reaction mixture was incubated at 70°C for 1 min to denature the vRNA, followed by immediate chilling in wet ice. Eighty units of avian myeloblastosis virus reverse transcriptase (from Joseph Beard, Life Science Inc., St. Petersberg, Fla.) were added. The reaction mixture was incubated at 37°C for 2 hr. An additional 80 units of reverse transcriptase was then added and the incubation was continued for another 2 hr. The reaction mixture was then boiled for 3 min followed by immediate chilling in wet ice to denature the first strand cDNA from the RNA template. This mixture was added to a second 400μl reaction mixture for second strand cDNA synthesis and incubated at 15°C for 4 hr. The latter mixture contained 8mM $MgCl_2$, 0.14M KCl, 0.4M Hepes, pH6.9, 1mM each of dATP, dGTP, dTTP, 0.25mM dCTP, 80μCi/assay [^{3}H] dCTP (New England Nuclear Canada, Dorval, Que.), 2mg/ml purified BSA and 200U/ml of E. coli DNA polymerase 1 (Klenow fragment) (Boehringer Mannheim, Quebec). The incorporation of [^{3}H]dCTP into acid precipitable material was monitored as an indication of the extent of cDNA synthesis. The reaction was stopped by the addition of SDS to 0.1% and carrier yeast tRNA to 125μg/ml. The suspension was extracted with an equal volume of phenol (90% v/v in TNE i.e. 0.01M Tris-HCl, pH7.5, 0.1M NaCl, 0.001M EDTA). The aqueous phase was extracted with ether. The cDNA was precipitated by the addition of sodium acetate (pH7.5) to 0.3M and 2.5 volumes 95% ethanol, at -70°C. cDNA was pelleted by centrifugation at 12,500g for 15 min at 4°C and the dried pellet was resuspended in 100μl of 30mM sodium acetate (pH4.5), 0.3M NaCl, 3mM $ZnCl_2$, 2μg of carrier single-stranded DNA and 400U Sl nuclease (Miles Laboratories). Enzymatic digestion was carried out at room temperature for 60 min. The reaction was stopped by the addition of SDS to 0.1%. The suspension was extracted with phenol. The DNA solution was desalted on a Sephadex G-50 column (bed volume 6ml) and eluted with a solution of TNE and 0.1% SDS. DNA from the pooled excluded volume was precipitated by the addition of 2.5 volumes ethanol at -50°C.

B. Cloning of Pichinde cDNA into the Pst 1 site of pBR322 by homopolymer tailing

Pichinde cDNA was pelleted by centrifugation at 12,500g for 15 min at 4°C and resuspended in 100μl of 0.2M sodium cacodylate, 0.03M Tris-HCl (pH7.6), 0.1mM DTT, 10mM $CoCl_2$, 100U terminal deoxynucleotidyl-transferase (PL Biochemicals, Milwaukee, Wisconsin) and 100μCi[^{3}H]dCTP (New England Nuclear Canada, Dorval, Que.). After 30 min at 37°C, the number of dC residues added was estimated at 28. Addition of dG residues to 10μg PstI digested pBR322 was carried out as above except that the incubation time was reduced to 2 min. The number of dG residues added was estimated at 37. Each reaction mixture was extracted with phenol and desalted by Sephadex G-50 chromatography as previously described. The dC-tailed Pichinde DNA and dG-tailed pBR322 were pooled and precipitated with 2 volumes of ethanol at -50°C. DNA was pelleted by centrifugation at 16,000g for 30 min at 4°C. The pellet was resuspended in 20µl TE buffer (0.01M Tris-HCl, pH7.5, 0.001M EDTA) and annealed for 12 min at 51°C followed by gradual cooling to room temperature over a 3 hr period.

Since the number of dG and dC residues added to Pst I digested pBR322 and Pichinde cDNA respectively were not identical, we compensated for this by using the Klenow fragment of E. coli DNA polymerase 1 to fill in additional dG and dC residues in the gaps of the annealed recombinant plasmid. The reaction mixture consisting of 8mM $MgCl_2$, 0.1mM dGTP, 0.1mM dCTP, and 10U DNA polymerase 1 (Klenow fragment) was then incubated at 37°C for 30 min. This was followed by a ligation reaction which would further stabilize the recombinant plasmid by covalently sealing the insert to the vector. This reaction consisted of 0.4mM ATP, 66mM Tris-HCl pH7.6, 6.6mM $MgCl_2$, 10.0mM DTT and 3U T4 DNA ligase and was incubated at 4°C overnight.

C. Transformation of Escherichia coli LE392

150ml of a log-phase culture of E. coli LE392 in Luria broth and 0.4% glucose (OD_{600}=0.2-0.4) was centrifuged at 6,000g for 10 min at 4°C. All procedures were then carried out on ice except where indicated. The bacterial pellet was resuspended in 1/4th the volume of original culture of cold 50mM $CaCl_2$. After 20 min at 4°C, the suspension was pelleted by centrifugation and resuspended in 1/40th the volume of original culture of 50mM $CaCl_2$. Varying amounts of recombinant plasmid were added to 0.3ml aliquots of E. coli LE392. After vortexing, the suspension was kept on ice for 40 min followed by heating at 42°C for 2 min. Aliquots of 100, 10 and 1 μl of the bacterial suspension were plated in duplicate on tetracycline containing (10μg/ml) agar plates which were then incubated at 37°C overnight. Ampicillin-sensitive, tetracyline resistant ($Ap^S Tc^R$) transformants were later selected by plating duplicate transformants on ampicillin-containing (20μg/ml) agar plates. Since the yield of recombinant plasmid DNA was higher in the host E. coli HB101, the cloned Pichinde DNAs were later transferred to this host.

XVI. Purification of Plasmid DNA

$Ap^S Tc^R$ transformants were rapidly screened for the presence of recombinant plasmids by gel electrophoresis. Small preparations of plasmid DNA were purified according to the method of Birnboim and Doly (1979). Transformants containing plasmid DNA larger than the vector pBR322 were selected for further characterization of the cloned inserts by sequence homology. Preparative purification of plasmid DNA was by cesium chloride-ethidium bromide equilibrium density centrifugation.

XVII. Preparation of Pichinde cDNA Probes

Radioactively labelled cDNA probes were prepared in a 100μl reaction mixture containing 50mM Tris-HCl (pH8.3), 8mM $MgCl_2$, 0.5mM dithiothreitol, 1mM each of dATP, dGTP, dTTP, 1-4μg of RNA (either total Pichinde RNA, cellular ribosomal RNA, isolated Pichinde L RNA or isolated S RNA), 60-240μg oligodeoxyribonucleotide (random) primer, 300μg/ml bovine serum albumin, 100μg/ml actinomycin D and 100-150μCi α-^{32}P-dCTP. The reaction mixture was incubated at 70°C for 1 min followed by immediate chilling in wet ice. Twenty units of reverse transcriptase was added. The reaction mixture was incubated at 37°C for a total of 4 hr. After the first 2 hr of incubation, an additional 20 units of enzyme was added. Template RNA was degraded by the addition of NaOH to 0.3N followed by incubation at 70°C for 25 min. The mixture was then neutralized with HCl. Twenty micrograms of carrier tRNA was added and the mixture deproteinized with phenol. The aqueous phase was desalted by chromatography on a Sephadex G-50 column. DNA from the excluded volume was precipitated with 2.5 volumes of ethanol at -50°C. DNA was pelleted at 10,000g and resuspended in a small volume of autoclaved water.

XVIII. Dot Hybridization

Recombinant plasmids were screened by sequence homology to Pichinde vRNA using a dot hybridization procedure (Kafatos et al., 1979). Purified recombinant plasmid DNA (3μg-10μg) was linearized by digestion with Hind III and treated with 200μg/ml proteinase K in 50mM Tris HCl, pH7.5 and 0.2% SDS. After a 30 min incubation at 37°C, carrier tRNA was added and the sample was deproteinized with phenol. The DNA was denatured in 0.4N NaOH for 10 min at 80°C followed by quick chilling on ice. An equal volume of ice-cold 2M ammonium acetate and 0.04N NaOH was added. Nitrocellulose filters were washed in 1 X SSC (0.15M NaCl, 0.015M sodium citrate) then in a solution of 1M ammonium acetate and 0.02N NaOH. The filters were assembled in a Hybri-dot apparatus (BRL, Maryland) and 50μl aliquots of DNA were applied to the filters under gentle vacuum. The filters were rinsed in a solution of 1M ammonium acetate and 0.02N NaOH, air dried at room temperature and baked at 80°C for 2 hr. Filters were stored at 4°C within a sealed plastic bag.

The nitrocellulose filters were pre-hybridized with 50% formamide, 5 X SSC, 5 X Denhardt's solution (0.1% BSA, 0.1% polyvinyl pyrrolidine, 0.1% Ficoll-MW 400,000), 50mM $NaPO_4$, pH6.5 and 0.5mg/ml salmon sperm carrier DNA for 4-6 hr at 42°C. The radiolabelled cDNA probe was boiled for 5 min and quickly added to a hybridization mixture consisting of 50% formamide, 5 X SSC, 1 X Denhardt's solution, 10% dextran sulphate, 20mM NaPO4 pH6.5 and 0.1mg/ml salmon sperm DNA. The hybridization was carried out within a sealed plastic bag at 42°C overnight. The filter was then washed in 2 X SSC and 0.1% SDS, four times 5 min each, followed by washing in 0.1 X SSC and 0.1% SDS, three times 15 min each, at 50°. After drying at 80°C, the filter was overlaid with Kodak XAR-5 film sandwiched between two Dupont Cronex X-tra light intensifying screens for autoradiography at -70°C.

XIX. RNA Blot Analyses

The RNA blot procedure used is similar to that published by Thomas (1980). Pichinde RNA denatured by glyoxal and DMSO at 50°C for 1 hour (section V-B) and electrophoresed in agarose slab gels was transferred to nitrocellulose filters. Hybridization of nick-translated cloned DNA probes to the filters was carried out as described in the dot hybridization section (XVIII).

XX. Preparation of a 3' End-Specific S RNA Probe

The 3' termini of RNA extracted from purified Pichinde virus preparations were labelled using RNA ligase and [^{32}P]pCp (New England Nuclear Canada, Dorval, Que.) in a 50μl reaction mixture consisting of 4mM dithiothreitol, 50mM Hepes, pH7.5, 20mM $MgCl_2$, 10% DMSO, 10μg/ml BSA, 12μM ATP, 300μg/ml viral RNA, 200 units/ml RNA ligase (PL Biochemicals, Milwaukee, Wisconsin) and 250μCi/assay [^{32}P]pCp. After a 48 hr incubation at 4°C, the RNA was precipitated with 2M ammonium acetate pH6.8 and 2.5 volumes ethanol at -20°C overnight. RNA was pelleted by centrifugation at 10,000g and resuspended in a small volume of autoclaved water. Viral RNA was then separated by electrophoresis on a 1% gel of low melting point agarose in 10mM methylmercury hydroxide (Ramsingh _et al._, 1980). The vRNA bands were visualized after staining of the gel in 3μg/ml ethidium bromide in autoclaved water. The agarose band containing the S RNA was excised from the gel and the RNA recovered by melting the agarose at 70°C in 20mM Tris HCl pH8.0, 0.5% SDS, 10mM EDTA and 1mM β-2-mercaptoethanol. After adding carrier yeast tRNA, the mixture was extracted with phenol. The aqueous phase was precipitated with 1/10 volume 3M sodium acetate pH7.0 and 2.5 volumes ethanol at -20°C overnight. RNA was pelleted at 10,000g and resuspended in 100mM Tris-HCl pH9.5. Alkaline digestion was done at 95°C for 30 min followed by rapid chilling in wet ice. The probe was neutralized and quickly added to the hybridization solution (described in section XVIII). This 3' end-specific S RNA probe was used in a dot hybridization assay to screen cloned DNAs.

CHAPTER 3

SIZE ESTIMATION OF PICHINDE VIRAL RNA BY GEL ELECTROPHORESIS UNDER DIFFERENT DENATURING CONDITIONS

I. Introduction

The overall aim of this research project was to define the Pichinde viral genes and their corresponding gene products. A prerequisite of this objective is an understanding of the structural organization of the viral genome.

A survey of the literature on the RNAs of arenaviruses reveal that at least 2 genomic RNA species, L and S, are associated with purified virus preparations. Although additional RNA species such as the 15S and 20S RNAs have been observed, the nature of these RNAs is not clear. Furthermore, analyses of the molecular weights of the viral RNAs by gel electrophoresis under non-denatured conditions have yielded varying results. Since the viral RNAs have a high degree of secondary structure, stringent denaturation of the RNAs is necessary to obtain valid estimations of the MWs of the RNAs. Secondary structure must be minimized so that electrophoretic mobility becomes a linear function of $\log_{10}$ MW.

The first task therefore, was to determine the number of RNA species that was associated with purified Pichinde virus preparations and to obtain estimations of the MWs of these viral RNAs by gel electrophoresis after stringent denaturation of the RNAs.

The results of this study were published under the heading "Size estimation of Pichinde virus RNA by gel electrophoresis under denaturing conditions" in Intervirology vol. 14:31-36 (Ramsingh, Dimock, Rawls and Leung, 1980).

II. Results

A. Agarose gel electrophoresis of Pichinde viral RNA in 10mM methylmercury hydroxide

1. Determination of the number of genomic RNA species present in purified Pichinde virus

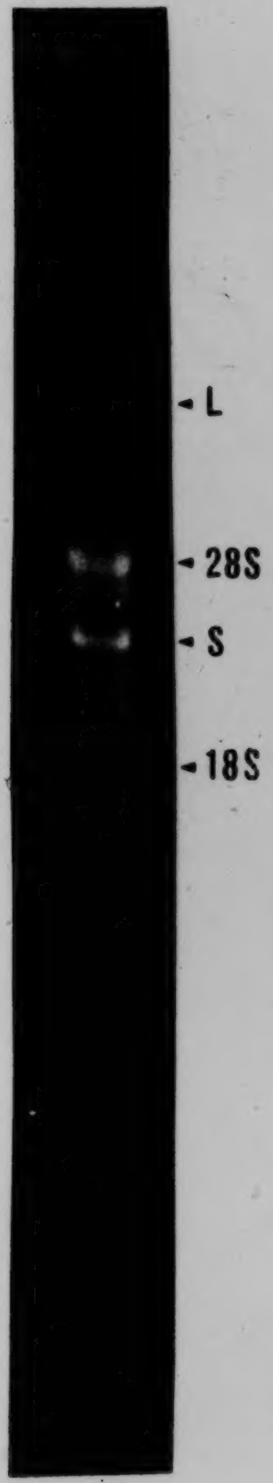
Pichinde virus, grown in BHK-21 cells, was radioactively labelled with [^{3}H]-uridine. RNA extracted from purified virus preparations was subjected to electrophoresis in agarose containing 10mM methylmercury hydroxide. When BHK-21 cells are infected at a low multiplicity of infection (0.1 pfu/cell) and virus is harvested early (24-72 hr post infection) in the infective cycle , the resulting RNA profile of this purified virus, visualized after gel electrophoresis and staining in ethidium bromide, yields 4 discrete RNA bands (Fig. 1a). In order to detect any minor RNA species not observed in the stained gels, the gels were cut into 2mm slices and processed for scintillation counting. A typical electropherogram is shown in Fig. 2. Three major and one minor peaks of radioactivity are observed. No significant amount of additional RNA species was detected by staining with ethidium bromide or after processing for scintillation counting.

When BHK-21 cells are infected at a low multiplicity of infection (0.1 pfu/cell) and virus is harvested late (72-120 hr post infection), the resulting RNA profile of this purified virus,

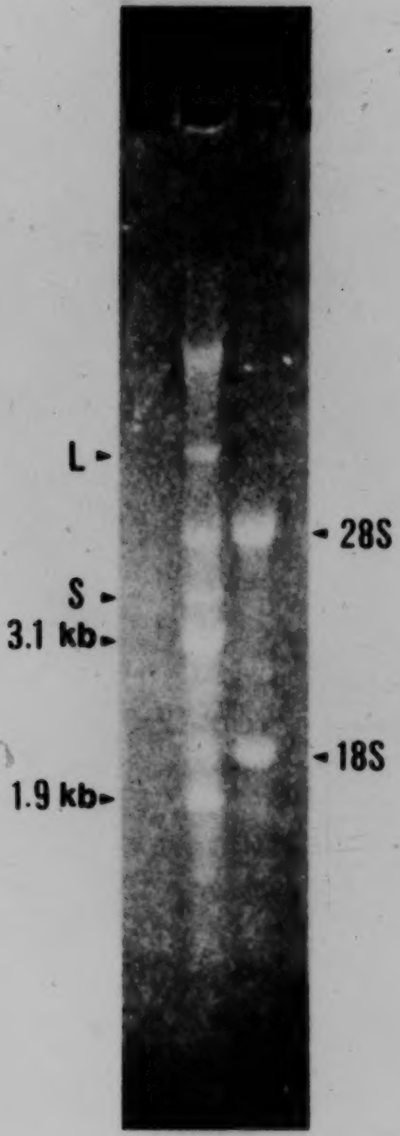
Fig. 1a. Ethidium-bromide stained gel profile of Pichinde viral RNA (obtained from virus harvested 24-72 hr post infection) after electrophoresis in 1% agarose containing 10mM methylmercury hydroxide.

Fig. 1b. A stained gel profile of Pichinde viral RNA extracted from virus harvested from 24 to 120 hr post infection (multiplicity of infection: 1pfu/cell).

(a)



(b)


L
S
3.1 kb
1.9 kb
28S
18S

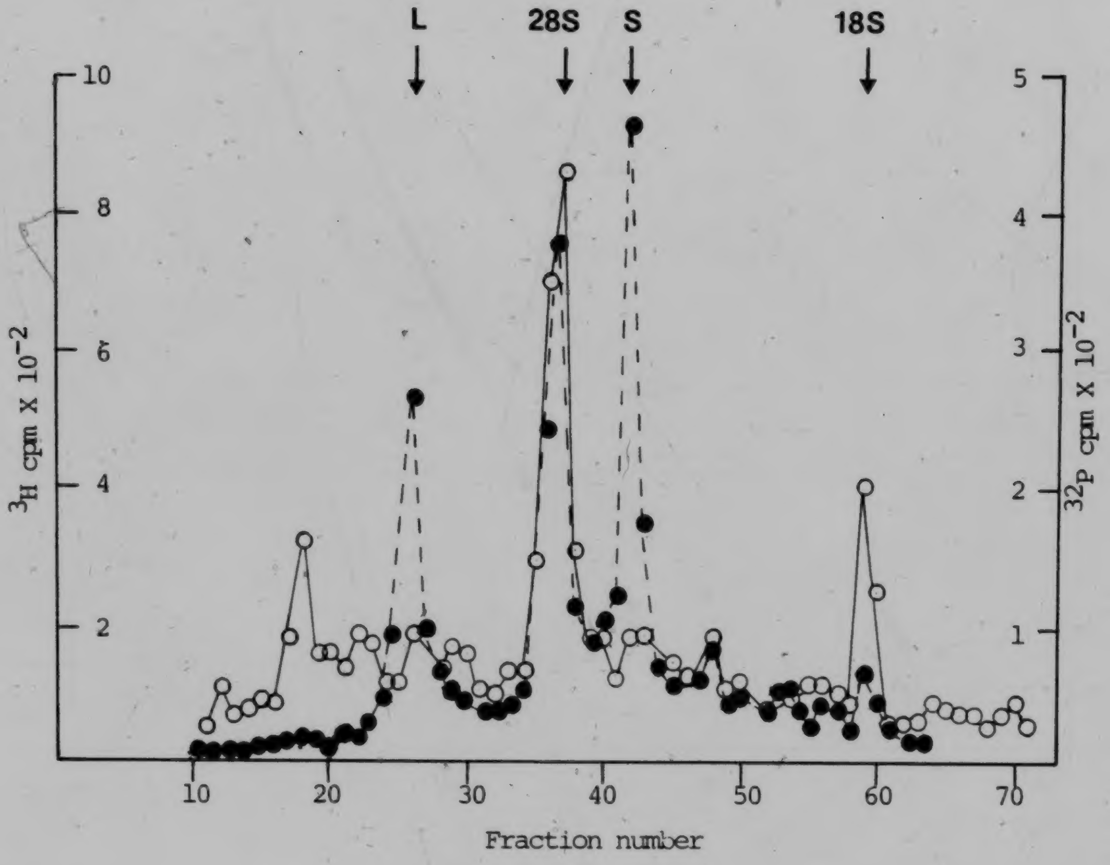


Fig. 2. Electropherograms of [^{3}H]-uridine-labelled Pichinde viral RNA (●- -●) and [^{32}P]-labelled BHK ribosomal RNA (○—○) after co-electrophoresis in a 1.5% agarose gel after denaturation in 10mM methylmercury hydroxide.

after gel electrophoresis and visualization by staining, yields 5 RNA species. The additional RNA species migrates slightly faster than the 18S ribosomal RNA in agarose gels containing methylmercury hydroxide. A better separation of these two RNAs can be observed when the Pichinde viral RNA is denatured by gloxyal-DMSO treatment and visualized by hybridization to a radioactive probe in RNA blot analyses (refer to Chapter 6).

When BHK-21 cells are infected at a high multiplicity of infection (1.0 pfu/cell) and virus is harvested and pooled from 24 hr to 120 hr post infection, the resulting RNA profile of this purified virus, visualized after gel electrophoresis and staining, yields 6 RNA bands i.e. the L and S viral RNAs, the 28S and 18S ribosomal RNAs and two additional RNA species designated 3.1kb RNA and 1.9kb RNA (Fig. 1b).

The RNA profile of purified Pichinde virus preparations seems to vary with both the time of viral harvest and the multiplicity of infection. Virus harvested early, after a low multiplicity of infection, was infectious as determined by a plaque assay in Vero cells. Further work on characterizing the genomic RNAs of Pichinde virus was therefore carried out with virus that was harvested 24 hr to 72 hr post infection after a multiplicity of infection of 0.1 pfu/cell.

Since Pichinde virus incorporates ribosomal RNAs into the progeny virions, the next step was to determine which RNA species represented viral genomic RNAs and which represented ribosomal

RNAs. This question was resolved by co-electrophoresis of 2 RNA samples i.e. ^{3}H-labelled Pichinde viral RNA and ^{32}P-labelled BHK-21 ribosomal RNA. Gels were sliced and processed for scintillation counting. A typical electropherogram of BHK ribosomal RNA is shown in Fig. 2. Two major peaks, representing 28S ribosomal RNA and 18S ribosomal RNA, are observed. Comparison of the electropherograms of Pichinde viral RNA and BHK ribosomal RNA reveal that 2 of the viral RNA species, 28S RNA and 18S RNA, comigrate with the 28S and 18S cellular ribosomal RNAs respectively. The other 2 viral RNA bands, L (large) RNA and S (small) RNA, therefore represent the viral genomic RNA species.

Although 4 to 6 RNA species were observed in purified Pichinde virus preparations, it appears that virus containing 4 RNA species is infectious. Two of these RNAs are ribosomal RNAs, 28S and 18S. Therefore, it seems that all of the genetic information of Pichinde virus, necessary to initiate a virus infection, resides in 2 RNA species i.e. the L and S RNAs. The nature of the additional RNAs will be discussed in later chapters.

2. Estimation of the molecular weight values of Pichinde viral L and S RNAs

Molecular weight estimates of the L and S RNAs were obtained from different gel concentrations to determine whether MW estimates were independent of agarose concentration. Three RNA markers were used: ^{3}H-labelled vesicular stomatitis viral RNA (MW 4 X 10^6), ^{32}P-labelled 28S ribosomal RNA (MW 1.7 X 10^6) and

^{32}P-labelled 18S ribosomal RNA (MW 7 X 10^5). Electropherograms of cellular ribosomal RNAs and VSV RNA are shown in Figs. 2 and 3 respectively. The electrophoretic mobilities of Pichinde viral L and S RNAs are thus well within the range of these 3 markers. Log molecular weight versus mobility plots were constructed for the 3 RNA markers electrophoresed in gels of different agarose concentrations i.e. 0.75%, 1.00%, 1.25% and 1.50% (Fig. 4). These plots are linear in the molecular weight range required (7 X 10^5 to 4 X 10^6 MW). MW estimates of Pichinde L and S RNAs were determined from these graphs. The MW values of the L and S RNAs range from 2.75 X 10^6 to 3.10 X 10^6 and from 1.12 X 10^6 to 1.45 X 10^6 respectively. Generally, the MW estimates are independent of gel concentration. However, the MW estimates at the highest gel concentration (1.50%) tend toward the upper limits of the MW ranges. Data from different experiments gave average MW estimates of 2.83 X 10^6 and 1.26 X 10^6 for the L and S RNAs respectively (Table 4). Additional verification of the validity of methylmercury-agarose gels for MW estimations is the absence of any obvious anomalies if the log mobility is plotted as a function of gel concentration (Ferguson, 1964) as shown in Fig. 5 where the data fall on straight lines intersecting at a common intercept at zero gel concentration.

3. Determination of the molar ratios of Pichinde L and S RNAs

The molar ratio of the L and S RNAs, present in an early harvest (24 - 48 hr post infection) of Pichinde virus, was

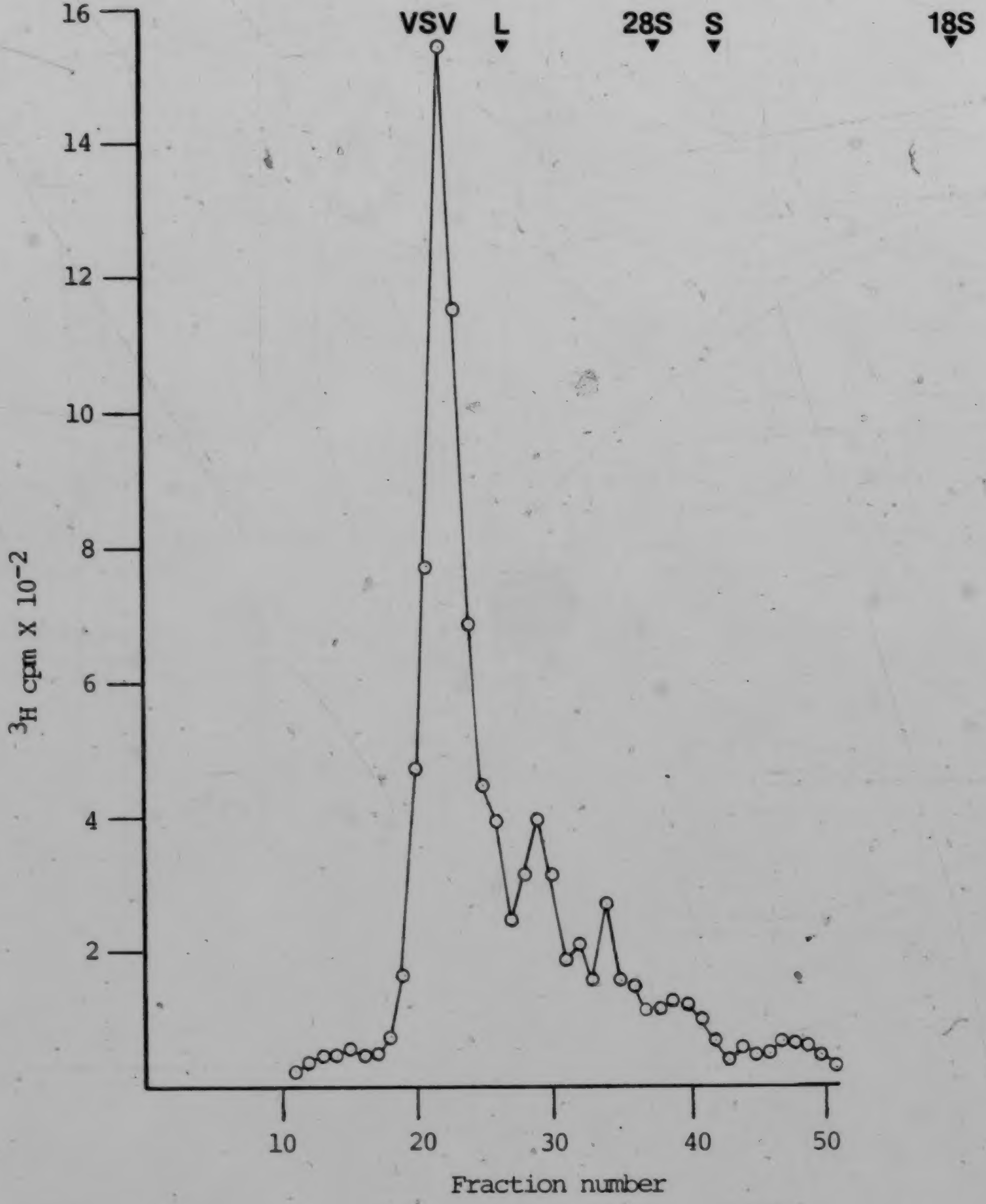


Fig. 3. Electropherogram of [^{3}H]-uridine-labelled VSV RNA in a 1.5% agarose gel after denaturation in 10mM methylmercury hydroxide.

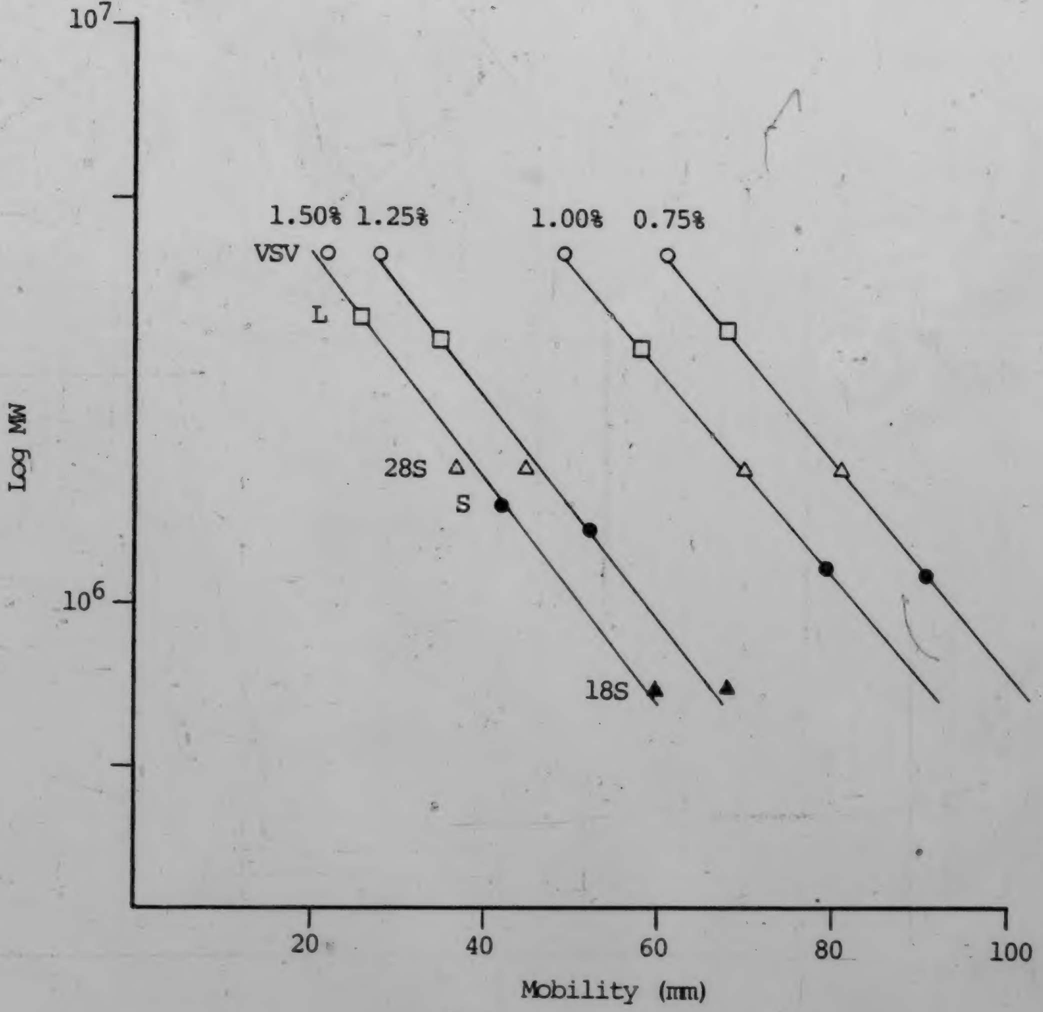


Fig. 4. Log of the MW versus mobility plots for Pichinde viral RNAs after electrophoresis in 10mM methylmercury hydroxide at 4 different agarose concentrations (0.75 , 1.00, 1.25 and 1.50%).

TABLE 4

Summary of MW determinations of Pichinde viral L and S RNAs after gel electrophoresis in 10mM methylmercury hydroxide

Pichinde RNA species	MW (X 10^6) estimations at various agarose gel concentrations			
	0.75%	1.00%	1.25%	1.50%
L RNA				
Experiment 1	2.70	2.70	2.90	2.70
Experiment 2	2.95	2.75	2.85	3.10
S RNA				
Experiment 1	1.20	1.20	1.30	1.35
Experiment 2	1.12	1.15	1.35	1.45

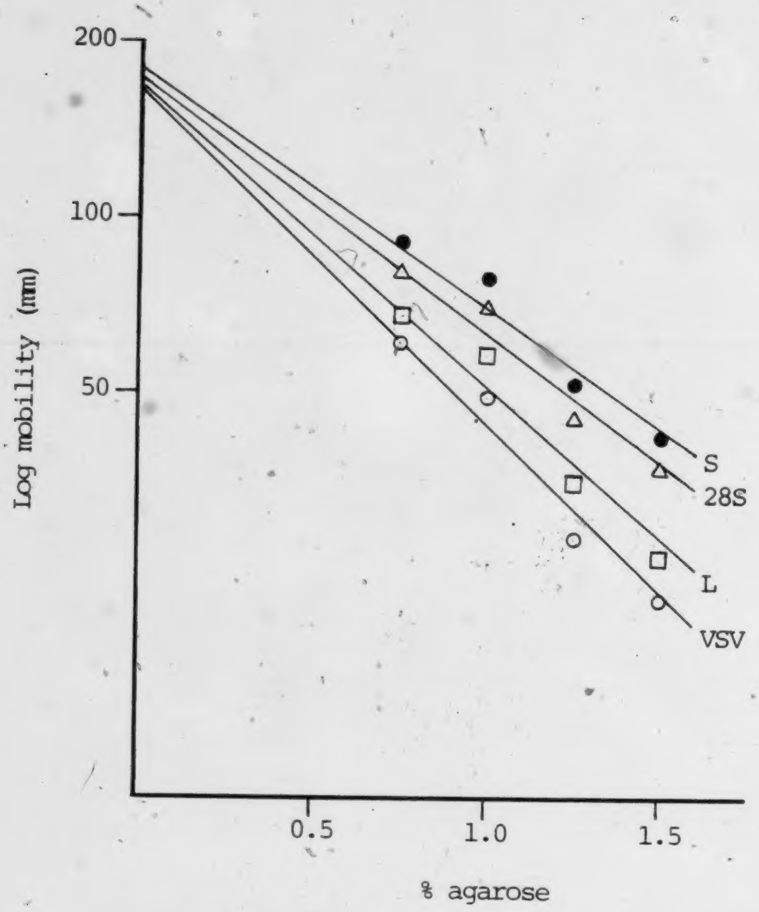


Fig. 5. Ferguson plots for Pichinde L and S RNAs, 28S ribosomal RNA and VSV RNA where log mobility is plotted as a function of the agarose concentration.

determined. Molar ratios were calculated using the MW values of the L and S RNAs and the peak areas (for L and S) obtained from electropherograms of viral RNA analyzed at the different agarose concentrations i.e. 1.00%, 1.25% and 1.50%. A summary of this data is shown in Table 5. The average molar ratio of the S RNA to the L RNA, in an early harvest of Pichinde virus, is 4:1. However, if virus is harvested late (72 to 120 hr post infection) in the infective cycle, the molar ratio of the S RNA to the L RNA is 6:1. Thus, the molar ratio of S RNA to L RNA varies with the time of harvest of virus.

B. Analysis of Pichinde viral RNA on polyacrylamide-agarose gels after denaturation with glyoxal and DMSO

Since VSV RNA is larger than Pichinde L RNA and both these RNA species are easily resolved by gel electrophoresis after denaturation in glyoxal and DMSO, Pichinde viral RNA and VSV RNA were co-electrophoresed in composite polyacrylamide-agarose gels. An acridine orange-stained gel profile is shown in Fig. 6. Five RNA species are seen. The largest RNA species represents VSV RNA while the remaining RNAs represent Pichinde RNA. Again, Pichinde viral RNA is resolved into 4 discrete bands: L, S, 28S and 18S. No additional RNA species was detected in stained gels. MW estimates of the L and S RNAs were determined at different acrylamide concentrations (from 1.0% to 1.6%) to determine if the MW estimates were independent of gel concentration. Log MW versus mobility plots were constructed for the 3 RNA markers (VSV RNA, 28S RNA, 18S RNA) electrophoresed in gels of different acrylamide

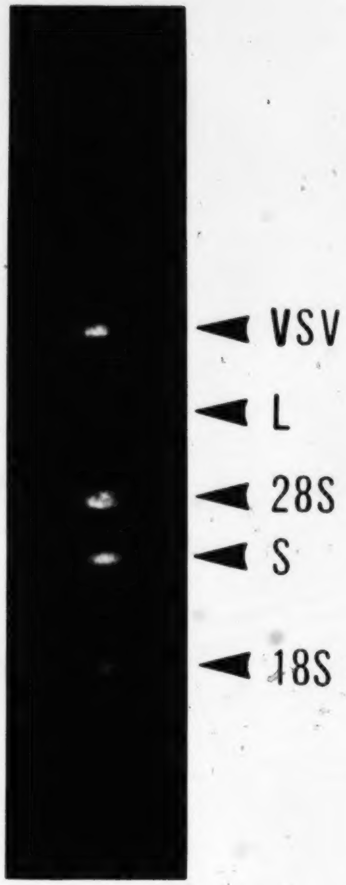
TABLE 5

Molar ratios of the L and S RNAs in an early harvest (24 to 72 hr post infection) of purified Pichinde virus

Agarose concentration (%)	RNA species	Area of peak (cm^2)	Ratio of area to *MW (X 10^6)	Molar ratio of S/L
1.00	S	1.89	1.50	3.8/1.0
	L	1.12	0.40	
1.25	S	3.63	2.88	4.0/1.0
	L	2.00	0.71	
1.50	S	5.22	4.14	4.1/1.0
	L	2.82	1.00	

*MW of S RNA : 1.26 X 10^6
MW of L RNA : 2.83 X 10^6

Fig. 6. Acridine-orange stained gel profile of Pichinde viral RNA and VSV RNA after denaturation in glyoxal and DMSO followed by co-electrophoresis in a 1% composite polyacrylamide-agarose gel.


VSV
L
28S
S
18S

concentrations (Fig. 7). These plots were linear in the molecular weight range used i.e. 7 X 10^5 - 4 X 10^6. The MW estimates of Pichinde L and S RNAs were determined from these graphs. The MW values of the L and S RNAs range from 2.50 X 10^6 to 2.90 X 10^6 and from 1.30 X 10^6 to 1.40 X 10^6 respectively. Generally, the MW estimates are independent of gel concentration. However, as shown above with methylmercury gels, the MW estimate of the L RNA is greater at the highest gel concentration (1.6%) than at lower gel concentrations. Data from different experiments gave average MW estimates of 2.63 X 10^6 and 1.31 X 10^6 for L and S RNAs respectively (Table 6).

C. Agarose gel electrophoresis of Pichinde viral RNA in 2.2M formaldehyde

The third denaturant used for electrophoretic analysis of Pichinde viral RNA was formaldehyde. Incubating the RNA in 2.2M formaldehyde and 20% formamide for 5 min at 60°C is generally sufficient for denaturation of large RNAs without appreciable degradation (Lehrach et al., 1977). This system was first tested using E. coli ribosomal RNA and BHK ribosomal RNA. The two RNA samples were co-electrophoresed in a 1% agarose gel and after staining in ethidium bromide, 4 RNA bands were clearly resolved: E. coli ribosomal RNAs, 23S and 16S, and BHK ribosomal RNAs, 28S and 18S. Similar analysis of VSV RNA revealed one discrete band. However, analysis of Pichinde viral RNA after denaturation in formaldehyde followed by electrophoresis in agarose gels of different concentrations (0.75%, 1.00%, 1.25% and 1.50%),

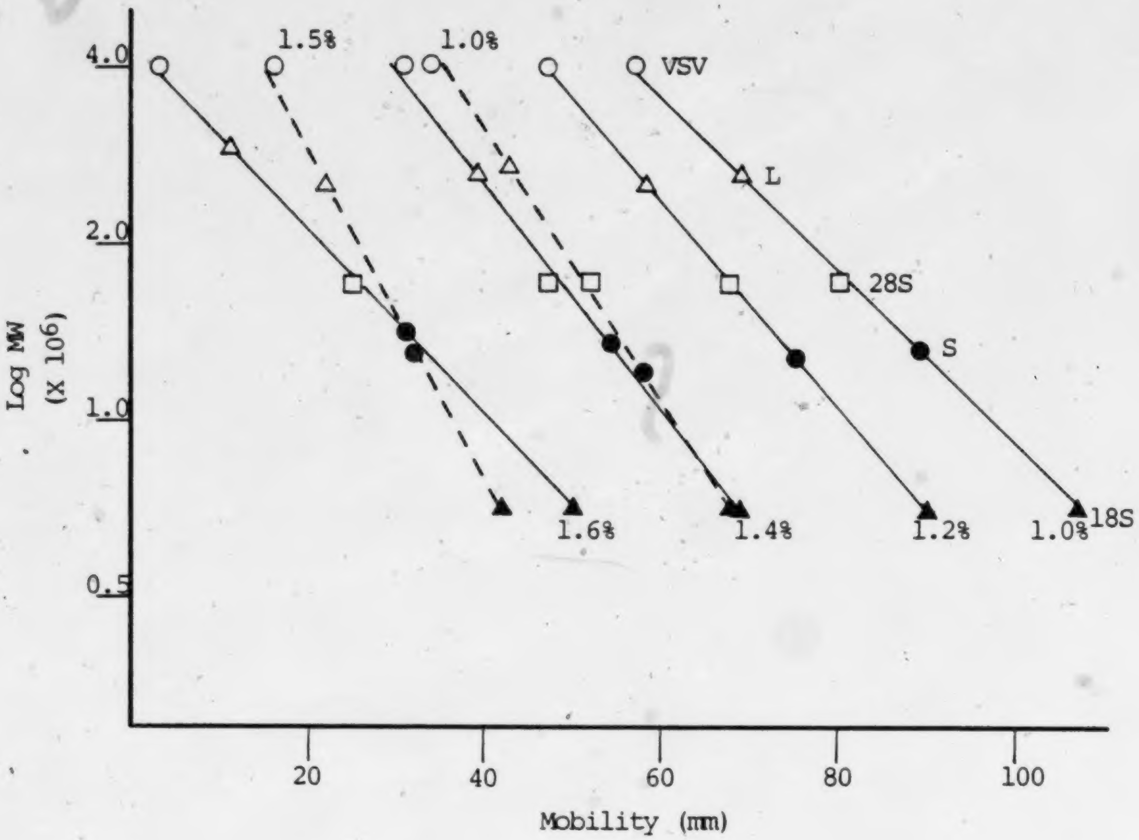


Fig. 7. Log of the MW versus mobility plots for Pichinde viral RNAs after denaturation in glyoxal and DMSO followed by electrophoresis in composite polyacrylamide-agarose gels. Acrylamide concentration was varied from 1.0% to 1.6%.

--- Results from experiment #1.

— Results from experiment #2.

TABLE 6

Summary of MW determinations of Pichinde viral L and S RNAs after denaturation in glyoxal and DMSO followed by electrophoresis in composite polyacrylamide-agarose gels

Pichinde RNA species	MW ($X 10^6$) estimations at various acrylamide concentrations				
	1.0%	1.2%	1.4%	1.5%	1.6%
L RNA					
Experiment 1	2.70	N.D.	N.D.	2.50	N.D.
Experiment 2	2.60	2.50	2.60	N.D.	2.90
S RNA					
Experiment 1	1.20	N.D.	N.D.	1.30	N.D.
Experiment 2	1.30	1.30	1.35	N.D.	1.40

N.D.: not done

revealed no discrete bands upon staining. A broad smear of fluorescence was observed implying that:

a) the Pichinde viral RNA preparation used had degraded prior to analysis, thus resulting in a smear of RNA fragments upon electrophoresis,

or

b) formaldehyde promotes cross linking between the different viral RNA species thereby resulting in aggregates that cannot be resolved upon gel electrophoresis.

To differentiate between these two possibilities, one preparation of Pichinde viral RNA was analyzed simultaneously using 2 denaturing systems i.e. formaldehyde and methylmercury hydroxide. Pichinde viral RNA, denatured in 10mM methylmercury hydroxide, was readily resolved into 4 bands: L, S, 28S and 18S. However, the same viral RNA preparation denatured in 2.2M formaldehyde revealed no discrete bands upon staining. Again, a broad smear of fluorescence was observed. These results imply that the Pichinde viral RNA preparation used, was intact and not degraded. Although formaldehyde readily denatures E. coli ribosomal RNA, BHK RNA and VSV RNA, it appears not to denature Pichinde viral RNA under similar conditions.

III. Discussion

The major aim of this research project was to define the Pichinde viral genes and their corresponding gene products.

Accurate estimations of the molecular weights of the viral genomic RNAs thus become important when attempts are made to localize the genes encoding the viral polypeptides.

Four RNA species, L, S, 28S and 18S, have been consisently observed in RNA extracted from purified preparations of Pichinde virus. Two additional RNA species, 15S and 20S, have occasionally been reported in the literature. However, the nature of these latter RNAs is unclear. Three RNA profiles were observed in our preparations of purified Pichinde virus. The RNA profile obtained was dependent on the time of viral harvest and the multiplicity of infection. Virus harvested early, 24 hr to 72 hr post infection, after a low multiplicity of infection (0.1 pfu/cell), yielded a typical RNA profile consisting of L, S, 28S and 18S RNAs. This virus was infectious as determined by a plaque assay. Since the 28S and 18S RNAs are of host cell origin, the remaining RNAs, L and S, are thought to be virus-specific. Thus, the genetic information of Pichinde virus appears to reside in a minimum of 2 RNA segments.

When BHK-21 cells are infected at a low multiplicity of infection (0.1 pfu/cell) and virus is harvested late (72 - 120 hr post infection), the resulting RNA profile of this purified virus yields 5 bands. The additional RNA species (0.6×10^6 MW, 1.9kb) may represent the 15S RNA that is occasionally observed in purified preparations of Pichinde virus (Farber and Rawls, 1975; Dutko et al., 1976). This 1.9kb RNA species does not appear necessary for the initiation of viral infection since viral preparations lacking this RNA species are infectious. The 1.9kb RNA is generated late in infection and may represent a truncated L or S RNA species. RNA extracted from DI virus preparations, obtained from a BHK cell culture persistently infected with Pichinde virus, lacked both the S and 15S RNAs, yet did contain the L RNA (Dutko et al., 1976). It is therefore possible that the 15S or 1.9kb RNA is related to the S RNA. However, the possibility of a cellular origin for the 1.9kb RNA cannot be excluded at this point.

When BHK-21 cells are infected at a high multiplicity of infection (1.0 pfu/cell) and virus is harvested and pooled from 24 hr to 120 hr post infection, the resulting RNA profile yields 6 bands, L, S, 28S, 18S, 1.9kb and 3.1kb (1.0 X 10^6MW). The 3.1kb RNA may represent the 20S RNA that has sometimes been seen in purified Pichinde virus preparations (Dimock et al., 1982; Dutko et al., 1976). Again, the 3.1kb RNA species does not appear necessary for viral infectivity. This RNA species seems to be generated after a high multiplicity of infection and thus may represent a defective viral RNA, originating from either the L or S RNA. Dutko et al. (1976) showed that a new 20S RNA species was observed in DI virus taken from cultures that had been maintained for more than 175 generations after the initial infection. The appearance of the 20S or 3.1kb RNA correlated with a progressive loss of the L RNA. This may indicate a relationship between the 3.1kb RNA and the L RNA. Another possibility is that the 3.1kb RNA is a virus-induced cellular RNA.

The previous estimates of the molecular weights for the L and S RNAs obtained by PAGE, varied from 3.1 X 10^6 (Vezza et al., 1978) to 2.1 X 10^6 (Carter et al., 1973) for the L RNA and from 1.6 X 10^6 (Vezza et al., 1978) to 1.1 X 10^6 (Carter et al., 1973) for the S RNA. These studies were conducted using heat-denatured or nondenatured RNAs. Since Pichinde viral RNA has considerable secondary structure as suggested by electron microscopic observations and resistance to RNase digestion, it seemed necessary to obtain MW estimations of the L and S RNAs by gel electrophoresis after denaturation of the RNA. Three denaturants were used, 10mM methylmercury hydroxide, glyoxal and DMSO and formaldehyde. The average estimates of the MW of S RNA are 1.26 X 10^6 using methylmercury hydroxide and 1.31 X 10^6 using glyoxal and DMSO. The MW estimates for L RNA are 2.83 X 10^6 using methylmercury hydroxide and 2.63 X 10^6 using glyoxal and DMSO. Although some variation is apparent, the average MWs determined for the L and S RNAs using methylmercury hydroxide or glyoxal and DMSO are in good agreement.

The MW estimates obtained following denaturation and electrophoresis of RNA in gels containing the highest concentration of acrylamide or agarose were slightly greater than those obtained from gels containing lower concentrations of acrylamide or agarose. The high values obtained by Vezza et al.

(1978) i.e. 3.2×10^6 and 1.6×10^6 for L and S, respectively, may partly be explained by the high concentration of acrylamide (2 - 2.5%) used in their gels.

An interesting observation was that although formaldehyde could readily denature E. coli ribosomal RNA, BHK RNA and VSV RNA, it could not denature Pichinde viral RNA under similar conditions. This may be a unique property of the RNAs of arenaviruses. After denaturation of Pichinde viral RNA in formaldehyde and analysis by gel electrophoresis, no discrete RNA bands was observed. Instead, a broad smear of RNA was seen. The viral RNA was intact as discerned by parallel analysis of viral RNA using methylmercury hydroxide as the denaturant. Since VSV RNA, BHK ribosomal RNA and E. coli RNA were denatured in formaldehyde while Pichinde viral RNA was not denatured under similar conditions, one possibility is that Pichinde viral RNA possesses more secondary structure than the other RNAs and hence requires more stringent denaturation. Another possibility is that formaldehyde promotes cross-linking between the various RNAs and the resulting complexes are visualized as a smear upon gel electrophoresis. In this case, cross-linking would occur between the viral RNAs (L, S) and the ribosomal RNAs (28S, 18S).

CHAPTER 4

DETERMINATION OF THE RELATIONSHIP BETWEEN THE L AND S RNAs OF PICHINDE VIRUS BY HYBRIDIZATION TO cDNA PROBES

I. Introduction

Having confirmed that the genome of Pichinde virus consists of 2 RNA species, L and S, and that the MW estimates of the L and S RNAs are 2.63 - 2.83 X 10^6 and 1.26 - 1.31 X 10^6 respectively, the next objective was to obtain an estimate of the gene coding capacity of this virus. The gene coding capacity would be different if both RNAs contain unique sequences or if the S RNA is a subset of the L RNA. The nucleotide sequence relationship between the L and S RNAs was examined by synthesizing cDNA from individual L and S RNAs for use as molecular probes in nucleic acid hybridization experiments. Previous studies showed that cDNA transcription can be restricted to a small region of the template RNA, primarily due to the secondary structure of the RNA. Since cDNA covering most or all of the template RNA was necessary for this study, it was important to first optimize the conditions required for synthesis of cDNA.

The results of this study have been published under the heading "Pichinde virus L and S RNAs contain unique sequences" in J. Virol. vol. 37: 48-54 (Leung, Ramsingh, Dimock, Rawls, Petrovich and Leung, 1981).

II. Results

A. Generation of random DNA primers for use in cDNA synthesis

Using the method of Taylor et al. (1976), oligodeoxyribonucleotides were generated for use as random DNA primers by limited DNase I digestion of calf thymus DNA. The oligodeoxynucleotides were fractionated on a Sephadex G-50 column and the gel filtration profile is shown in Fig. 8. The excluded volume in fractions 1 to 5 contained polynucleotides larger than 20 nucleotides. Two peaks are observed in the gel filtration profile. The first peak, between fractions 15 and 50, is broad and heterogeneous. The second peak, between fractions 50 and 60, consisted of mononucleotides. The oligonucleotide fractions were pooled into 6 groups (A to F) and assayed for priming activity in a reverse transcriptase reaction. Without the addition of primer, negligible amounts of DNA was synthesized (Fig. 9). Of the 6 groups of oligonucleotides tested, groups C and D exhibited the highest amount of priming activity as monitored by incorporation of ^{3}H-dGTP in a reverse transcriptase assay. Group C and D primers were used in subsequent cDNA syntheses. The estimated average lengths of oligonucleotides in groups C and D were 10 and 7 respectively. Oligonucleotides larger than 10 and smaller than 7 exhibited decreased primer function for Pichinde viral RNA.

The optimal ratio between the DNA primer and Pichinde viral RNA was investigated (Fig. 10). Maximal primer function was observed when the DNA primer to RNA template ratio ranged

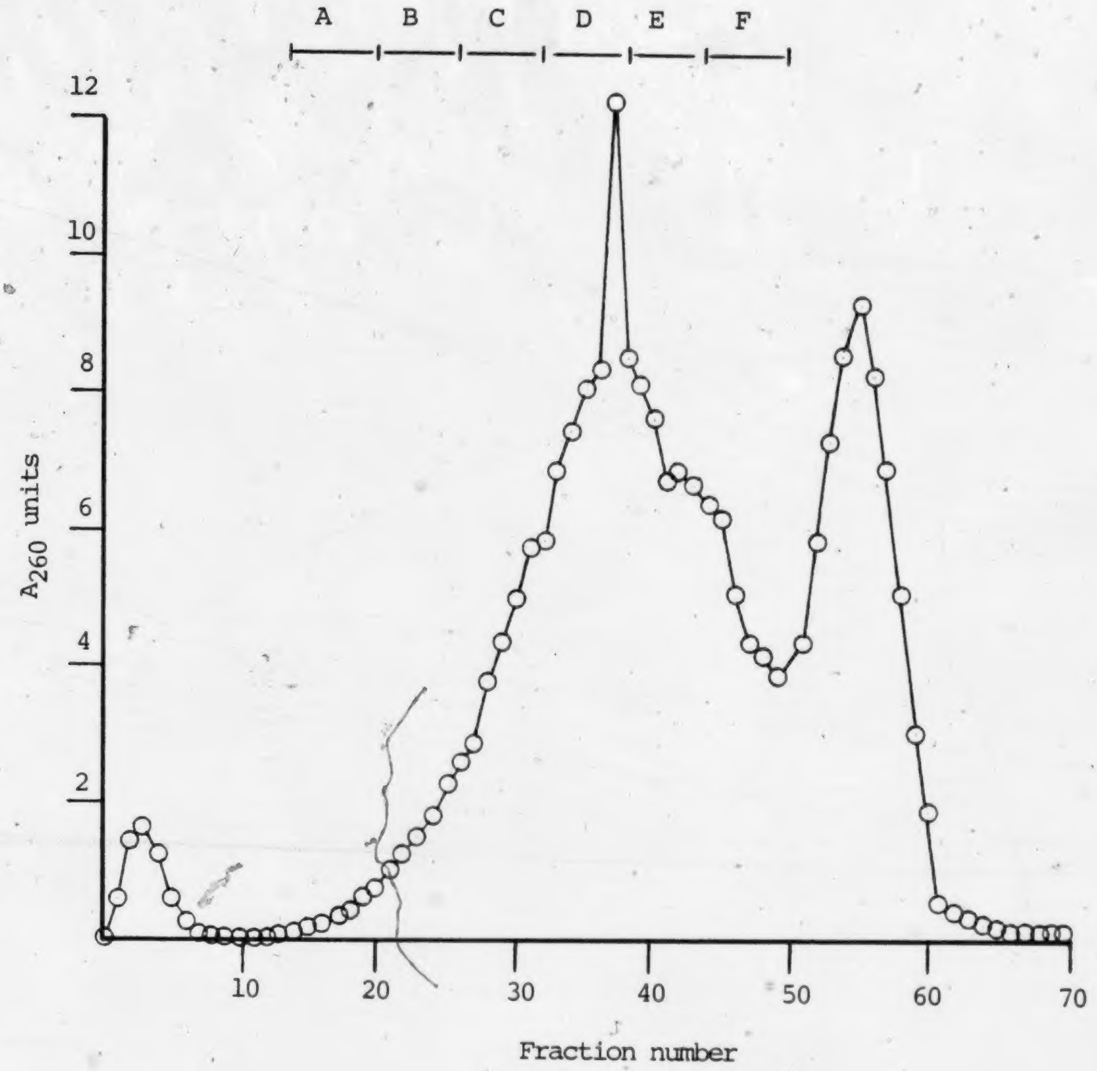


Fig. 8. Gel filtration profile of oligodeoxynucleotides after fractionation on a Sephadex G-50 column (Leung et al., 1981).

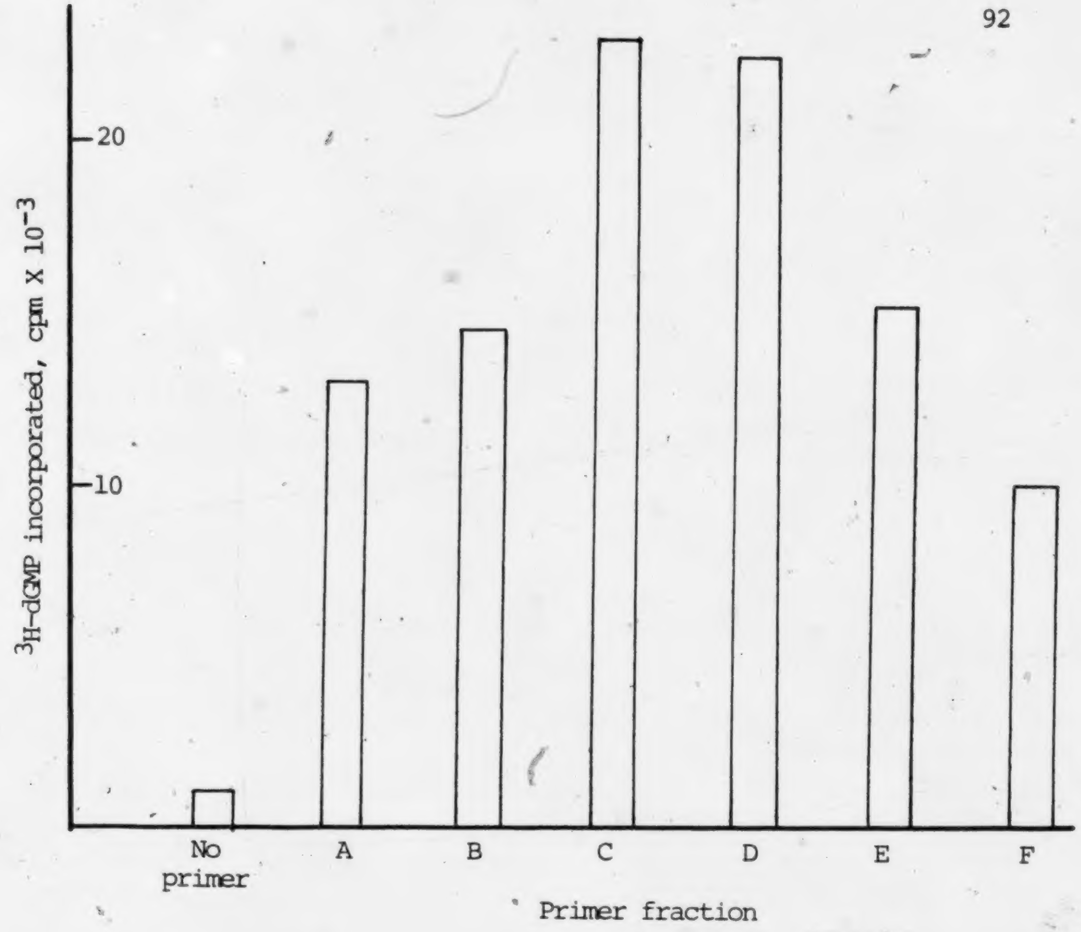


Fig. 9. Effect of different size classes of oligodeoxynucleotides on *in vitro* cDNA synthesis (Leung *et al*., 1981).

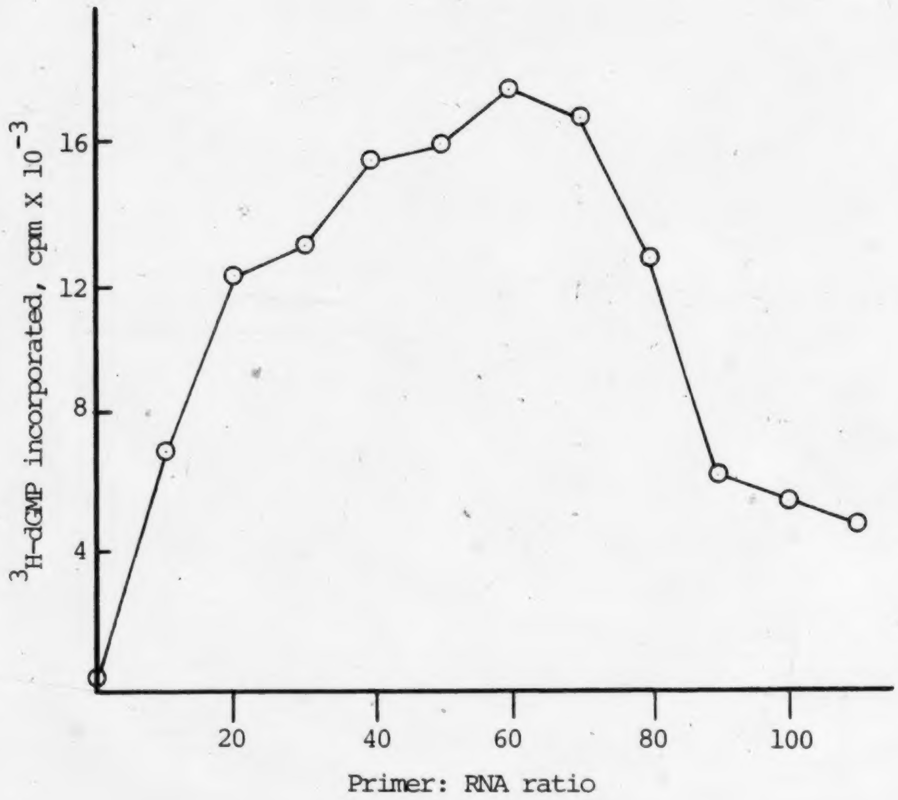


Fig. 10. Effect of oligodeoxynucleotide primer-to-RNA template ratio on *in vitro* cDNA synthesis (Leung *et al*., 1981).

from 40:1 to 70:1. A ratio of 60:1 was used for subsequent cDNA syntheses.

B. Conditions for cDNA synthesis

Complementary DNAs from individual L and S RNAs of Pichinde virus were required as molecular hybridization probes to examine the relationship between the L and S RNA species. Since we required transcription of most or all of the L and S RNA templates, we chose to optimize the conditions for *in vitro* cDNA synthesis. Each of the following components of the reaction mixture was examined at various concentrations to determine the concentration which yielded optimum DNA synthesis viz. KCl, DTT, BSA, actinomycin D, template RNA, reverse transcriptase and cold dCTP. Acid-precipitable radioactivity was used to monitor cDNA synthesis. In addition, cDNA products were size-fractionated by gel electrophoresis after denaturation in 10mM methylmercury hydroxide. The range of size classes of cDNA products did not vary appreciably when the components of the cDNA reaction mixture were examined at various concentrations. cDNA synthesis was therefore monitored, primarily, by the incorporation of [^{3}H]-dCMP into acid-precipitable material.

Maximum cDNA synthesis was observed at KCl concentrations of either 0mM or 35mM, whereas inhibition was observed beyond 70mM (Fig. 11). KCl was therefore omitted from the reaction mixture in subsequent experiments. Maximum cDNA synthesis was observed at a DTT concentration of 0.5mM (Fig. 12). Inhibition was

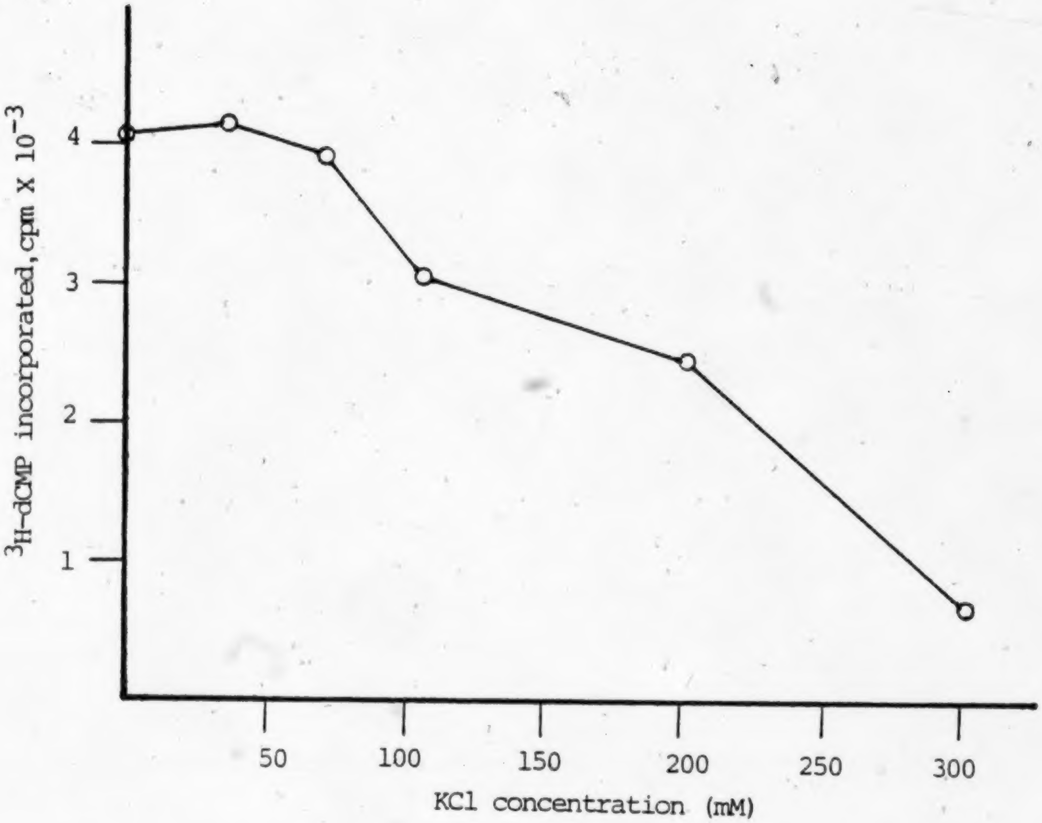


Fig. 11. Effect of KCl concentration on cDNA synthesis.

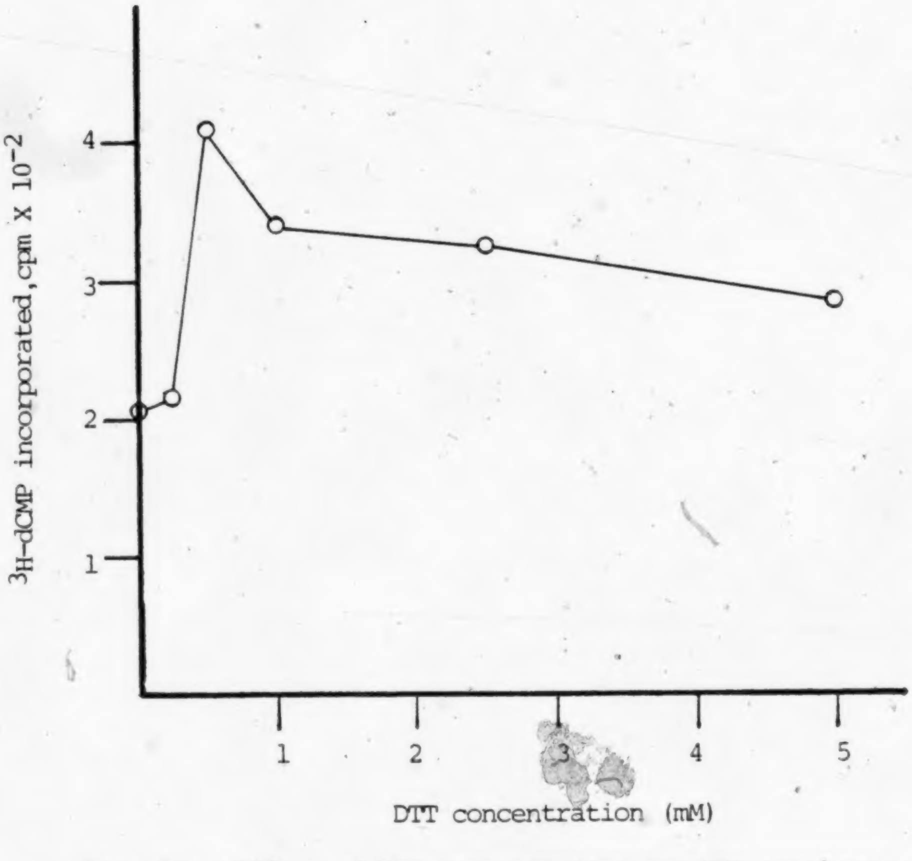


Fig. 12. Effect of DTT concentration on cDNA synthesis.

observed at concentrations greater than or less than 0.5mM. A DTT concentration of 0.5mM was used in later experiments. The results shown in Fig. 13 demonstrate that the optimal concentration of carrier protein BSA is 300μg/ml. Since the BSA preparation had been purified through UMP-agarose to remove possible contaminating RNase, the observed inhibitory effect at concentrations greater than 300μg/ml was probably due to increased viscosity of the reaction mixture. The results in Fig. 14 show that the optimal concentration of actinomycin D is 0 - 10μg/ml. Addition of actinomycin D to the cDNA reaction mix should increase the probability of generating longer single-stranded cDNA products by preventing synthesis of the second DNA strand. However, analysis of the cDNA products by gel electrophoresis under denaturing conditions (methylmercury hydroxide denaturation) revealed no differences in the range of size classes of cDNA made in the presence or absence of actinomycin D. However, without actinomycin D, more of the smaller cDNA products were seen. Since single-stranded cDNA was required as a hybridization probe, second-strand DNA synthesis was minimized by using 100μg of actinomycin D per ml of reaction mix. A template RNA concentration of 20μg/ml was selected for subsequent studies since the reaction rate began to plateau beyond this concentration (Fig. 15). The concentration of reverse transcriptase which gave maximal activity was 200U/ml (Fig. 16). Low levels of RNase activity were apparently present in the reverse transcriptase preparation

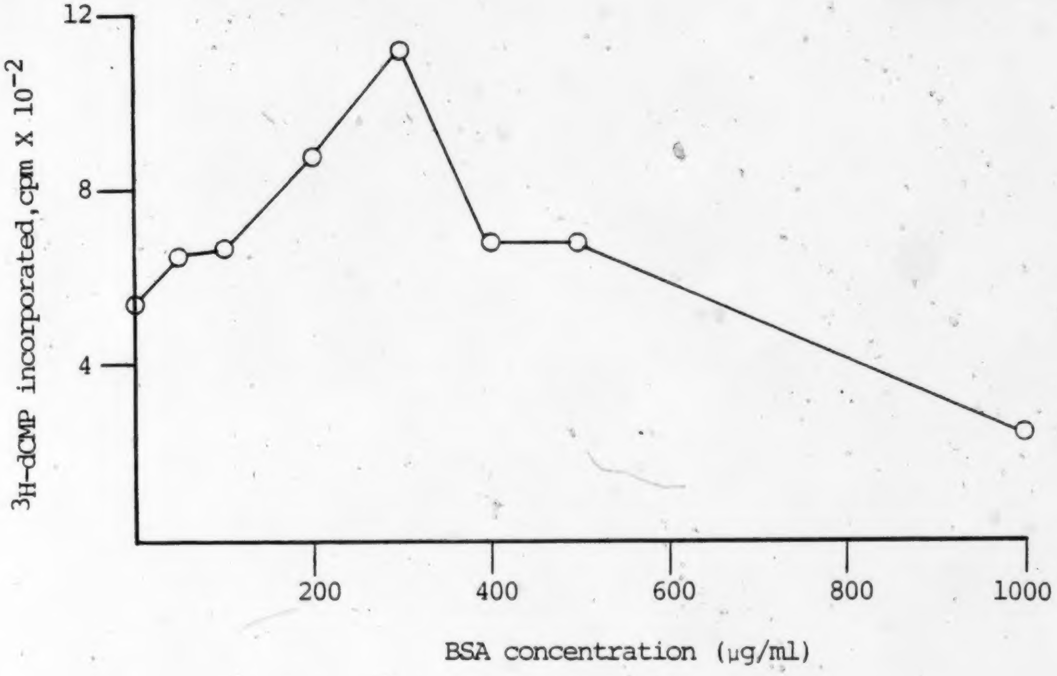


Fig. 13. Effect of BSA concentration on cDNA synthesis.

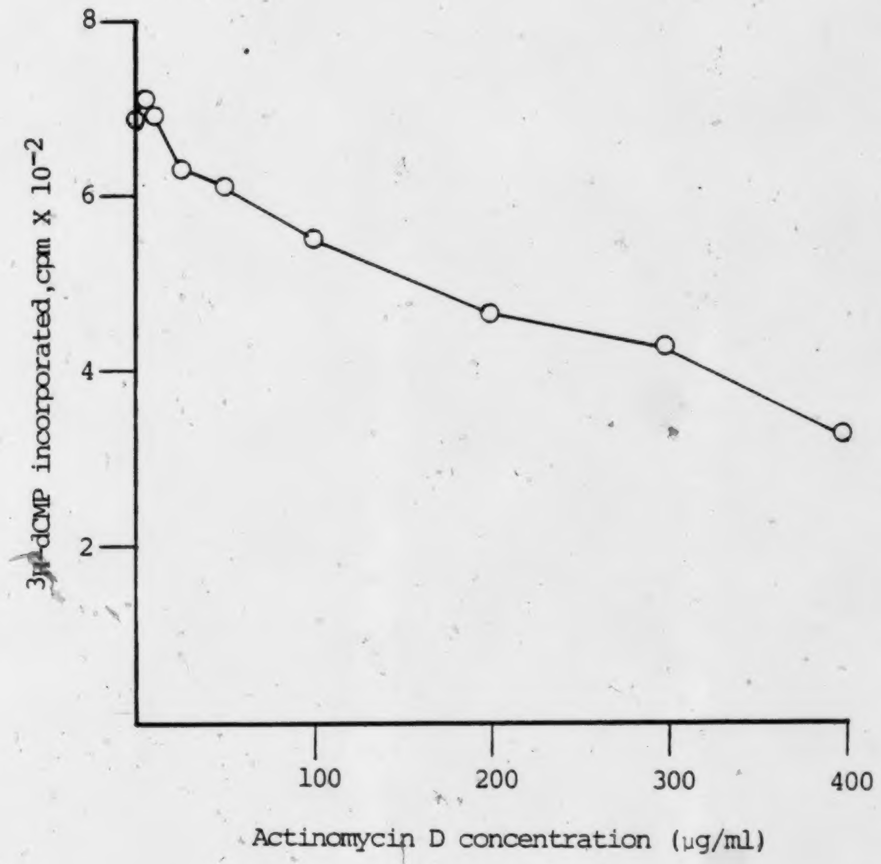


Fig. 14. Effect of actinomycin D concentration on cDNA synthesis.

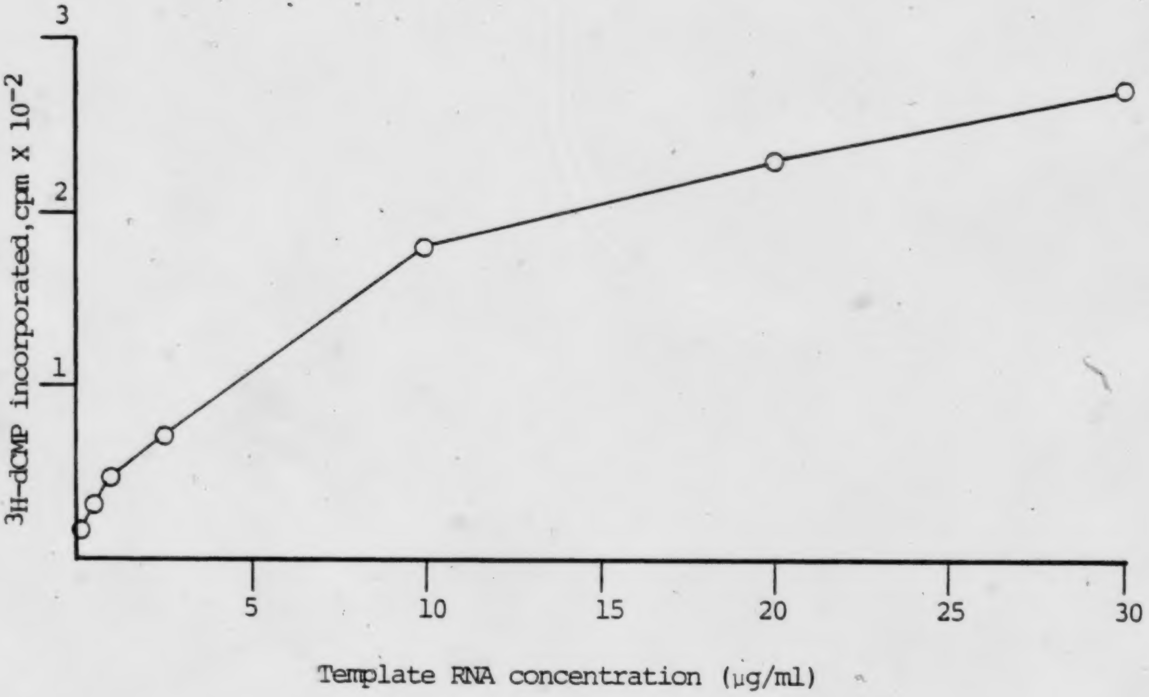


Fig. 15. Effect of template RNA concentration on cDNA synthesis.

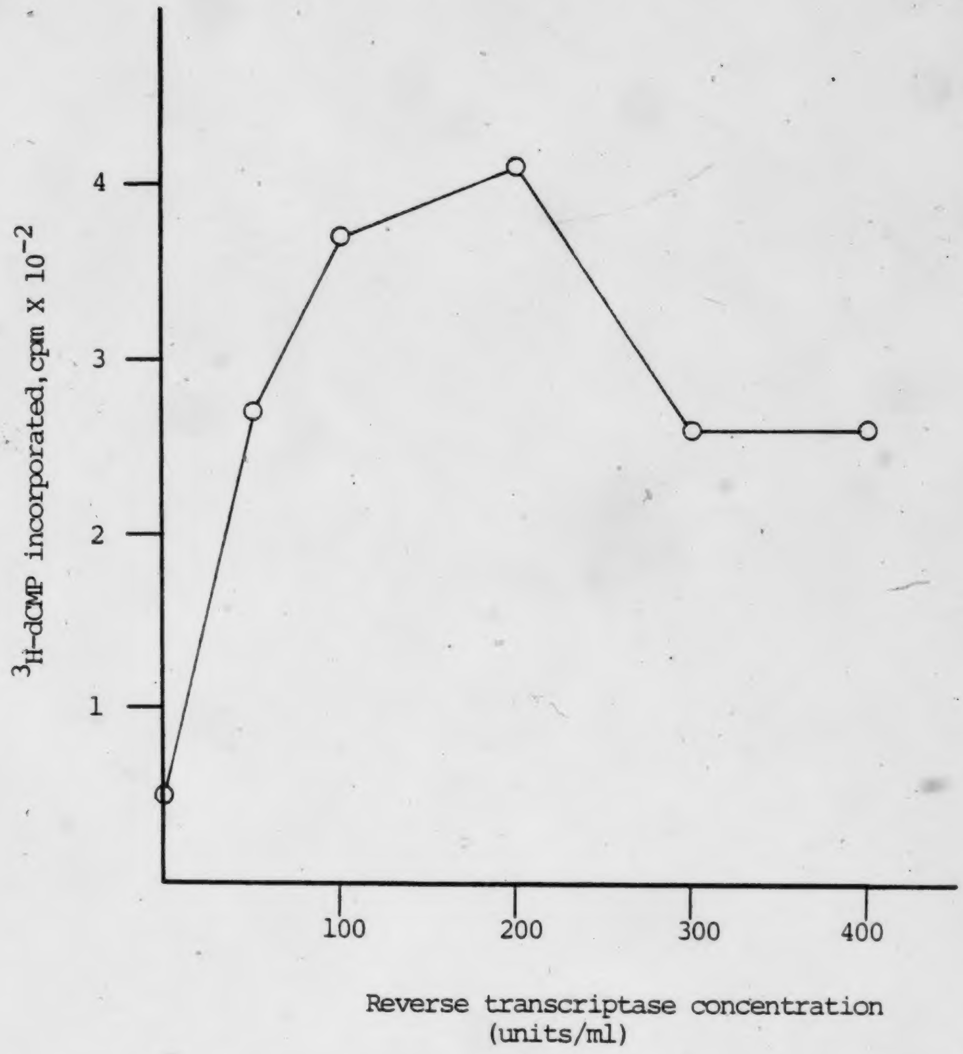


Fig. 16. Effect of reverse transcriptase concentration on cDNA synthesis.

(J. Beard, personal communication) and it is possible that the contaminating RNase activity was responsible for the decrease in DNA synthesis at higher enzyme concentrations. Three cold deoxynucleotide triphosphates (dATP, dGTP, dTTP) and one radioactively labelled deoxynucleotide (dCTP) were used to make cDNA probes since this combination resulted in the synthesis of the most radioactively labelled cDNA (Fig. 17). However, in later experiments when the amount of cDNA synthesized was important, cold dCTP was used at a concentration of 0.25mM.

The final conditions used for making Pichinde cDNA probes were as follows: 50mM Tris-HCl, pH 8.3, 8mM $MgCl_2$, 1mM dATP, 1mM dGTP, 1mM dTTP, 5μCi/assay $[^3H]$-dCTP, 0mM KCl, 0.5mM DTT, 300μg/ml BSA, 100μg/ml actinomycin D, 20μg/ml template RNA, 1200μg/ml oligodeoxynucleotide primer (60:1 primer to template ratio) and 200 units/ml reverse transcriptase.

C. cDNA synthesis from individual L and S RNAs

Using the optimum conditions for Pichinde cDNA synthesis, extensive reverse transcription occurred throughout the L and S RNAs (Leung _et_ _al_., 1981). 90% of L RNA was protected from RNase digestion by L cDNA while 92% of S RNA was protected from RNase digestion by S cDNA. The cDNAs were then used to probe for sequence homology between the L and S RNAs. 89% of the L cDNA hybridized with L RNA while 90% of the S cDNA hybridized with S RNA (Figs. 18, 19). 3 - 5% hybridization was observed when either L cDNA was hybridized to S RNA or when S cDNA was hybridized to L RNA. This amount of reannealing was similar to that observed

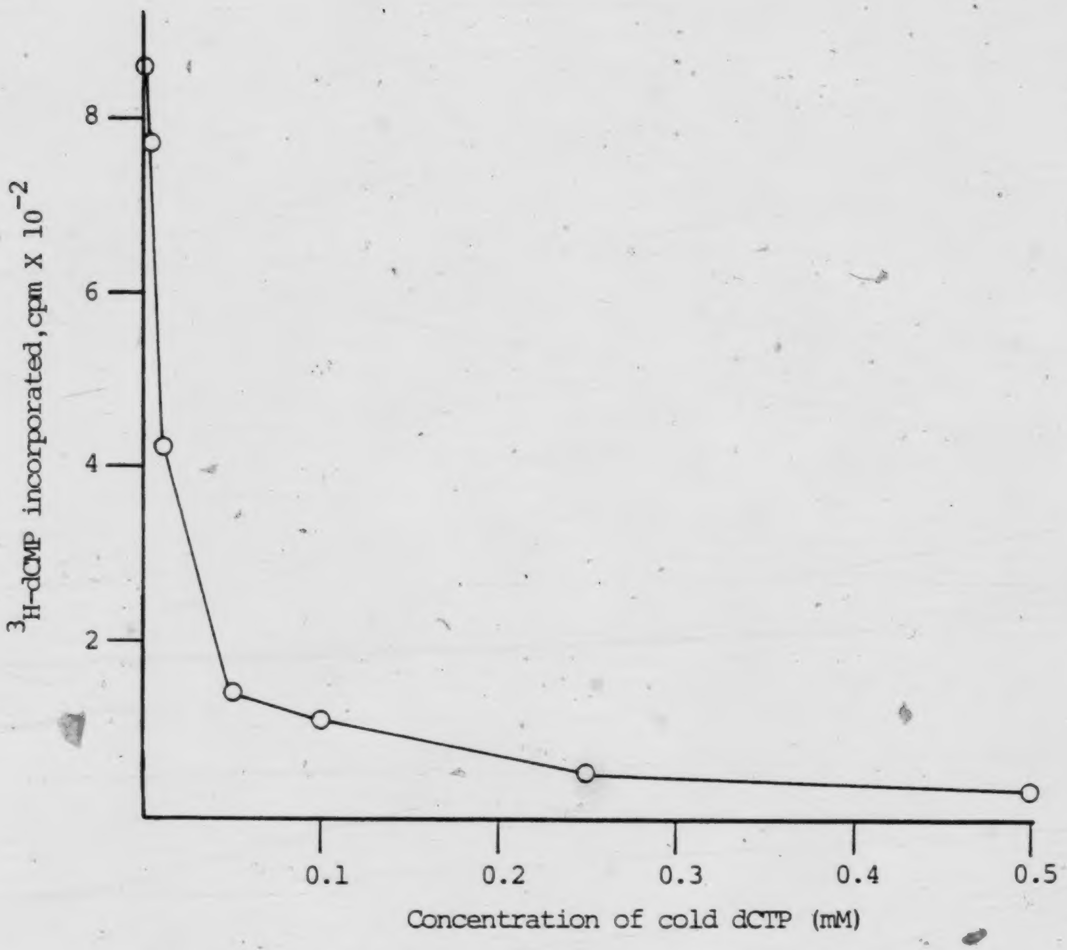


Fig. 17. Effect of various concentrations of cold dCTP on cDNA synthesis.

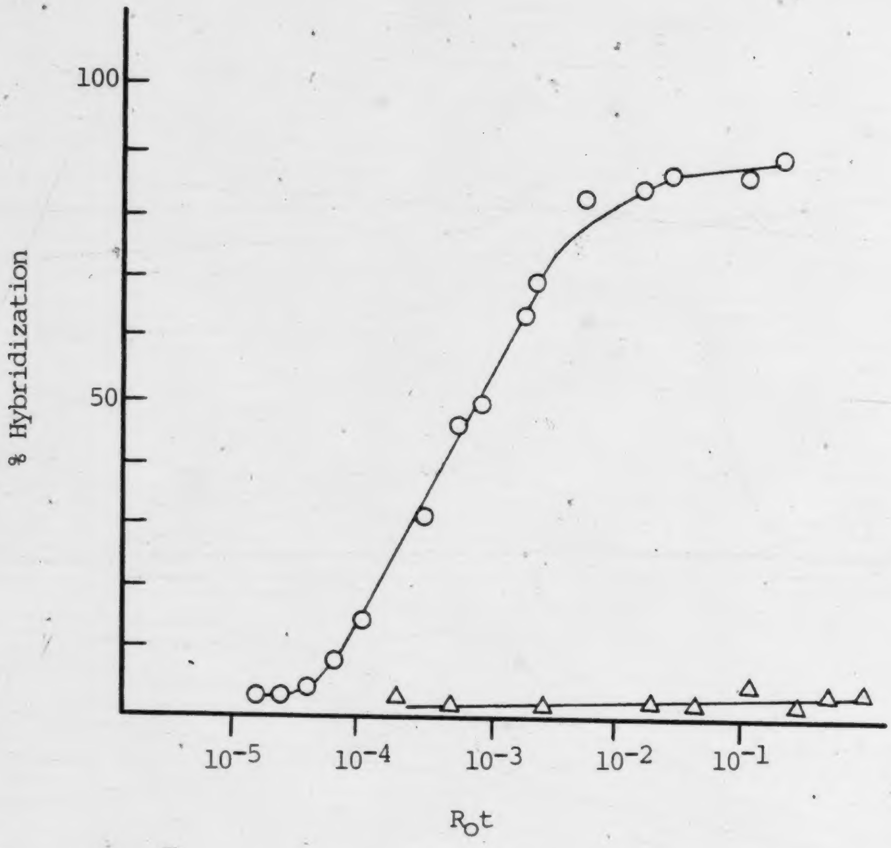


Fig. 18. Hybridization of [^{3}H]-L cDNA with Pichinde viral L or S RNA.

L RNA ○—○

S RNA △—△

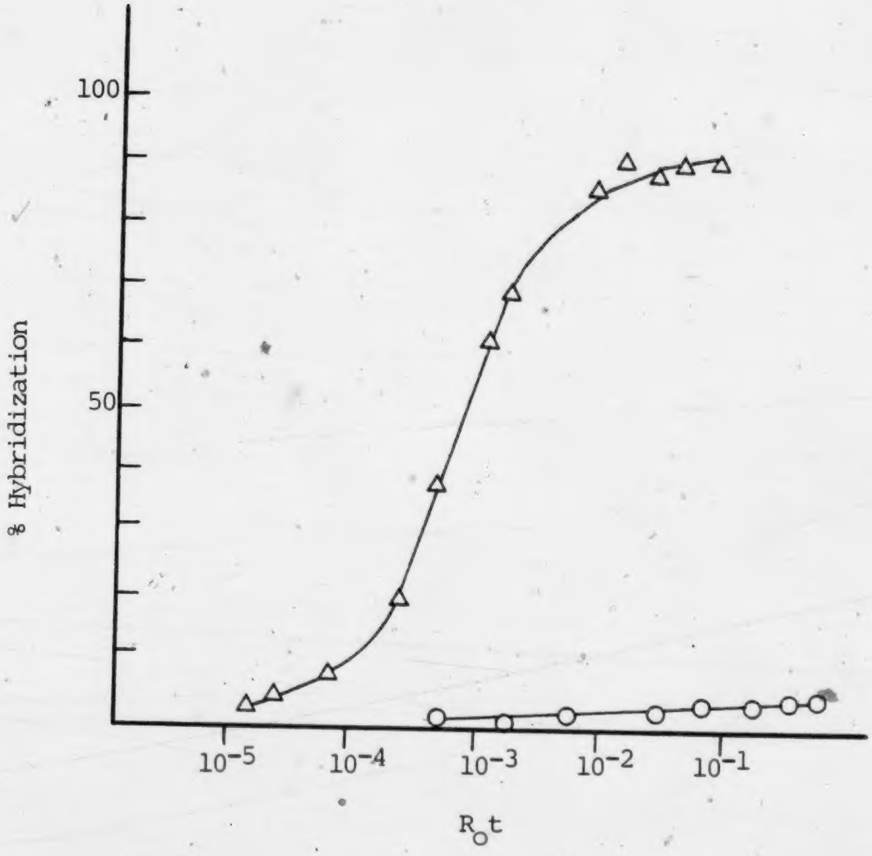


Fig. 19. Hybridization of $[^3H]$-S cDNA with Pichinde viral L or S RNA.

S RNA △—△

L RNA ○—○

when either L cDNA or S cDNA self-reannealed in the absence of viral RNA.

III. Discussion

Sequence homology between the L and S RNAs of Pichinde virus was examined by liquid hybridization using cDNA probes prepared from isolated L or S RNA. The cDNA probes were generated using AMV reverse transcriptase and oligodeoxynucleotides derived from limited DNase I digestion of calf thymus DNA as primers for random initiation of cDNA synthesis along the RNA template. About 90% of both the L and S RNAs were transcribed using the conditions outlined, despite the fact that considerable secondary structure has been observed in Pichinde viral RNA (Vezza _et_ _al_. 1978). This extensive transcription of the viral RNAs by reverse transcriptase was achieved only when the various conditions for the reaction mixture had been optimized. An interesting feature of Pichinde cDNA synthesis is that primers of average length of 7 to 10 nucleotides provided optimal priming function. The optimal length of the primer used for Pichinde cDNA synthesis is shorter than the 9 to 14 nucleotide used to prime cDNA synthesis of immunoglobulin RNA (Stavenezer and Bishop, 1977). In contrast to the suggested concentration of KCl i.e. 70mM for cDNA synthesis of various templates, more Pichinde cDNA was synthesized at KCl concentrations of 0 and 35mM. Whether the lower amount of KCl required is a reflection of the secondary structure of Pichinde RNA is unclear. However, a similar effect of KCl concentration has also been observed for ovalbumin RNA (Monahan et al., 1976).

Using the cDNAs synthesized from individual L or S RNA as molecular probes, 89% of the L cDNA hybridized with L RNA and 90% of the S cDNA hybridized with S RNA. About 3 to 5% hybridization was observed when either L cDNA was hybridized to S RNA or when S cDNA was hybridized to L RNA. Since the cDNA probes were homologous to about 90% of the viral RNA templates, these results suggest that the L and S RNAs are mainly unique. A small amount of cross-hybridization was observed when L cDNA and S cDNA hybridized to S RNA and L RNA respectively. One possible interpretation of these results is that the L and S RNAs share a few common sequences. Another possibility is that the L and S cDNA probes were contaminated with S and L cDNA respectively. The S cDNA could have been contaminated with L cDNA since the S RNA template was isolated from a gel after electrophoresis. The isolated S RNA may have contained some degraded L RNA which could also have been transcribed by reverse transcriptase. An analogous argument cannot be used to explain contamination of the L cDNA probe with S cDNA since the template L RNA isolated from a gel after electrophoresis is very pure. It thus seems likely that although the L and S RNAs contain mainly unique sequences, these 2 RNAs do share a few common sequences.

The L and S RNAs of Pichinde virus are mainly unique. This result agrees with the observations that a high recombination rate is observed between temperature-sensitive mutants of Pichinde virus (Vezza and Bishop, 1977) and the oligonucleotide fingerprints obtained for the L and S RNAs are different (Vezza *et al*., 1978). All of this information strongly suggest that the L and S RNAs of Pichinde virus code for different genetic information.

CHAPTER 5

GENE MAPPING OF THE PICHINDE VIRAL GENOME

I. Introduction

One of the primary goals of this research was to assign the viral genes to the Pichinde viral genome. Our first approach was to estimate the potential coding capacity of the viral genome which consists of the L and S RNAs. Since only 3 primary gene products had been detected in virus-infected cells, one could tentatively assign the 3 polypeptides to the 2 genomic RNAs based on the potential coding capacity data. A second approach (Leung _et_ _al_., 1981; Harnish _et_ _al_., 1983) involved the use of a recombinant virus Re-2 which contained the L RNA of Pichinde virus and the S RNA of Munchique virus, a related arenavirus. Analysis of the polypeptides of Re-2 and comparison with that of the parental viruses, Pichinde and Munchique, allowed the assignment of virus-specific polypeptides to the individual L or S RNA. A third approach involved the use of an antiserum to a synthetic peptide to define the polypeptide encoded at the 3' proximal end of the S RNA. The success of this approach allows the fine mapping of the gene products at the nucleotide sequence level.

II. Results

A. Gene assignment based on the potential coding capacity of the viral genome

Having estimated the size of the viral RNAs and having shown that the L and S RNA species are mainly unique, one could now calculate the potential coding capacity of the viral genome. Assuming the simplest method of encoding the viral polypeptides i.e. non-overlapping reading frames without splicing events, one could make the following calculations. The average MWs of the L and S RNAs have been estimated at 2.73×10^6 and 1.28×10^6 respectively. The L and S RNAs could therefore potentially encode polypeptides of MW 2.8×10^5 and 1.3×10^5 respectively (Table 7).

Three primary viral gene products have been detected by immunoprecipitation studies in Pichinde virus-infected cells (Harnish et al., 1981). These 3 polypeptides are a large protein, L, (MW approx. 200,000), a nucleoprotein, NP, (MW 64,000) and a precursor glycoprotein, pGP-C, (MW 42,000). The precursor glycoprotein gives rise to the glycosylated product, GPC, (MW 79,000) which is subsequently cleaved to yield 2 virion envelope glycopeptides, GP-1 (MW 55,000) and GP-2 (MW 36,000).

The gene assignment for the 3 primary gene products can be deduced as follows. The size of the L polypeptide exceeds the coding capacity of the S RNA but can easily be encoded by the L RNA (Table 8). The combined molecular weights of pGP-C and NP is 1.1×10^5 which could easily be encoded by the S RNA (Table 8).

TABLE 7

Estimation of the potential coding capacity of the Pichinde viral genome

	Average MW	Estimated no. of bases[1]	Potential number of amino acids encoded	Estimated coding capacity[2] (MW)
L RNA	2.73×10^6	8.5×10^3	2.8×10^3	2.8×10^5
S RNA	1.28×10^6	4.0×10^3	1.3×10^3	1.3×10^5

[1] assuming the average molecular weight of one nucleotide is 320

[2] assuming the average molecular weight of one amino acid is 100

TABLE 8

Assignment of the polypeptides to the Pichinde viral genome on the basis of size

RNA species	Estimated coding capacity (MW)	Primary gene products	MW's of primary gene products	
L RNA	2.8×10^5	L	2×10^5	
S RNA	1.3×10^5	PGP-C	4.2×10^4	total of 1.1×10^5
		NP	6.4×10^4	

However, because of the large size of the L polypeptide, one cannot accurately determine the MW of this protein by SDS-PAGE. The value of 200,000 as the MW of the L protein is only an approximation. Due to this uncertainty, the L RNA may encode the L protein and additional proteins such as NP and/or pGP-C.

At this point in time, our general hypothesis for the gene mapping of Pichinde virus is that the L RNA codes for the L protein while the S RNA codes for the NP and pGP-C. This hypothesis was tested using a recombinant virus, Re-2, which was provided by D. Bishop (Birmingham, Alabama) (Leung et al., 1981; Harnish et al., 1983). This virus had previously been used to show that the S RNA codes for the NP (Vezza et al., 1980; Compans et al., 1981).

Re-2 contains the L RNA of Pichinde virus and the S RNA of Munchique virus (a related arenavirus). The virus-specific polypeptides of Re-2 were analyzed by immune precipitation of infected cell extracts. The NP of Re-2 had an electrophoretic mobility similar to that of wild type (wt) Munchique virus but different from that of wt Pichinde virus. Since Re-2 and wt Munchique virus share a common S RNA, this suggests that NP is encoded by the S RNA (Vezza et al., 1980). Since no apparent difference exists in the electrophoretic mobilities of GP-C of wt Pichinde and wt Munchique viruses, the ^{35}S-methionine tryptic peptides of GP-C were compared. At the same time, tryptic peptides of NP were also compared. The peptide profile of GP-C of Re-2 was similar to that of wt Munchique but different from that of wt Pichinde virus. Similarly, the peptide profile of NP of Re-2 was similar to that of wt Munchique but different from that of wt Pichinde virus.

These results therefore support the model that both NP and pGP-C are encoded by the S RNA (Leung et al., 1981; Harnish et al., 1983). Tryptic peptide analyses of the L proteins of Re-2, Pichinde and Munchique showed that the gene locus for the L protein is on the L RNA (Harnish et al., 1983). A major problem of this approach is the isolation of sufficient amounts of radioactively-labelled L protein. However, as discussed previously, since the L protein exceeds the coding capacity of the S RNA, the L protein must be encoded by the L RNA.

B. Orientation of the genes on the S RNA

The experimental evidence thus far, shows that both the NP and the pGP-C map on the S RNA. The next task was to orient these two genes on the S RNA so that a fine map of the genome could be obtained. The sequence of the first 120 nucleotides at the 3' terminus of the S RNA had been determined (Auperin et al., 1982). The first UAC triplet on the genomic RNA (corresponding to the AUG start site on the mRNA) identified in this region was at residues 84-86. The corresponding amino acid sequence encoded at residues 84 to 120 was deduced (Fig. 20). The approach used to orient the NP and pGP-C genes on the S RNA involved synthesizing a peptide, ser-asp-asn-ile-pro-ser-phe-arg,

```
       1
3'  HO G C G U G U C A C C U A G G A U C C G C U G U G A U C U A G U G C
                                                          60
       G A C A U G C A A G U G A A G A A A U G A C U G A G U C U C C U U
                                        84
       C A C A C U U G U U G A G G U U U U A C A G G C U G U U A U A G
                                          met  [ser   asp   asn   ile
                                                120
       G G U A G C A A G G C G A C C C A C G U U A
        pro   ser   phe  arg]   trp   val   gln
```

Fig. 20. Nucleotide sequence of the 3' terminus of the S RNA of Pichinde virus (Auperin *et al.*, 1982).

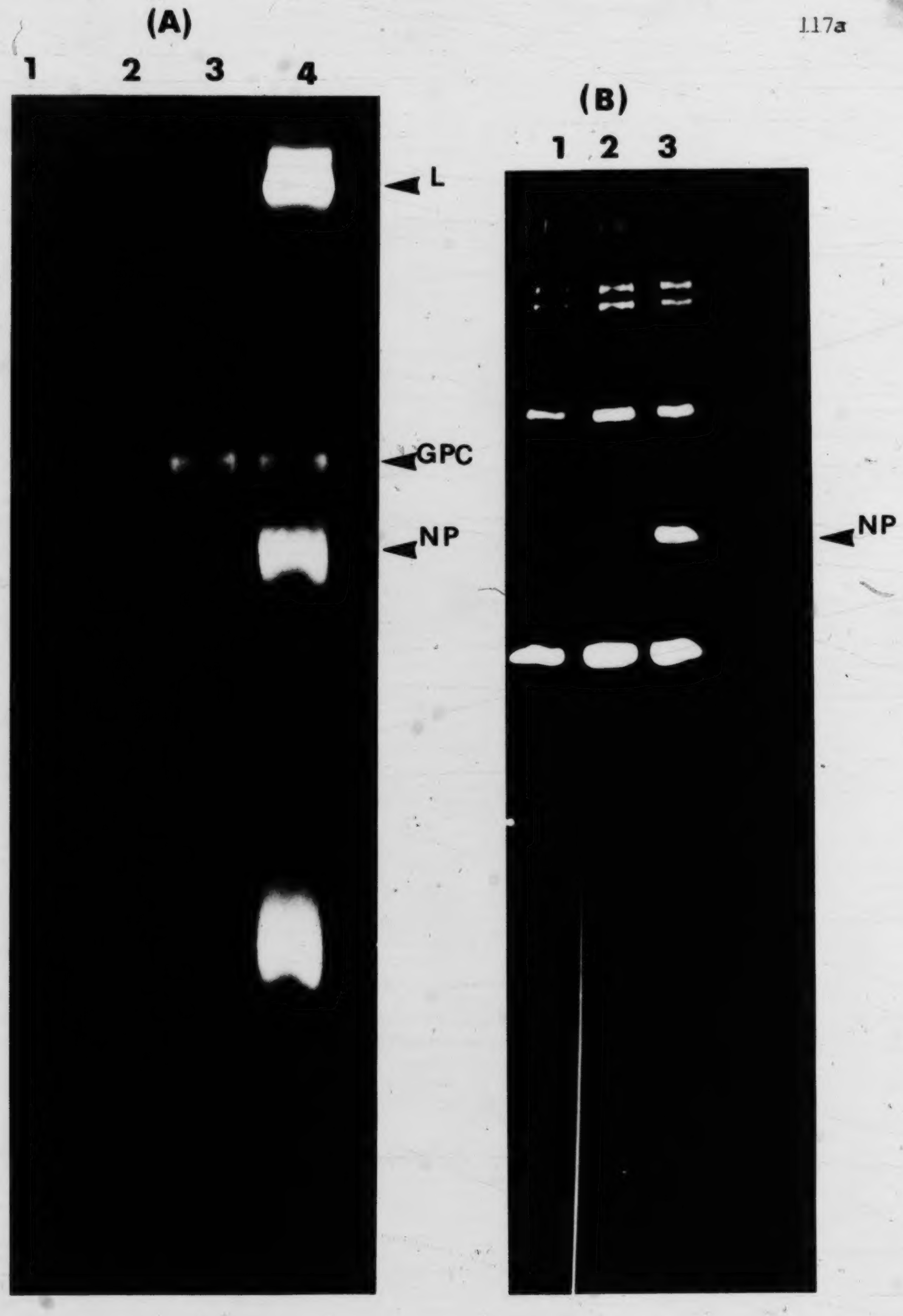
(R. Hodges and A. Akoki, unpublished data) corresponding to the first 8 amino acids encoded in the 3' terminus of the S RNA. This peptide was coupled to an immunogen, BSA and used to raise an antiserum which could identify the polypeptide encoded at the 3' terminus of the S RNA by immunoprecipitation.

1. Immunoprecipitable polypeptides in BHK cells

Pichinde virus-specific polypeptides in BHK cells were detected by immunoprecipitation using a hamster anti-Pichinde serum. Cells were infected at a multiplicity of infection of 10 pfu/cell and labelled with L-[^{35}S] methionine at 21 to 24hr post infection. Four major immunoprecipitable polypeptides were detected in virus-infected cells i.e. L protein, GP-C, NP and a 38K protein (Fig. 21a). The 38K protein is apparently related to the NP (Harnish et al., 1981). These proteins were not detected in mock-infected cell lysates or in infected cell lysates immunoprecipitated with normal hamster serum. An interesting observation not previously reported, is that the L protein is synthesized in relatively large amounts in infected BHK cells. This protein was readily detected if after cell lysis, cellular debris was removed by centrifugation at 10,000 g for 15 min. However, if after cell lysis, cellular debris was removed by centrifugation at 100,000 g for 1 hr, very little L protein was detected. There was also a concomitant reduction in the amount of NP and NP38 detected (Fig. 21a).

Fig. 21a. PAGE analysis of immunoprecipitated Pichinde virus-specific polypeptides from BHK cells. Lane 1: lysate of infected cells precipitated with non-immune serum. Lane 2: lysate of uninfected cells precipitated with hamster anti-Pichinde serum. Lane 3: lysate of infected cells precipitated with hamster anti-Pichinde serum after a clarifying spin at 100,000g. Lane 4: lysate of infected cells precipitated with hamster anti-Pichinde serum after a clarifying spin at 10,000g.

Fig. 21b. PAGE analysis of immunoprecipitated Pichinde virus-specific polypeptides from BHK cells. Lane 1: lysate of uninfected cells precipitated with hamster anti-Pichinde serum. Lane 2: lysate of infected cells precipitated with rabbit pre-immune serum. Lane 3: lysate of infected cells precipitated with rabbit antiserum to a Pichinde synthetic peptide coupled to BSA.


(A)
1
2
3
4
L
GPC
NP
(B)
1
2
3
NP

2. Immunoprecipitation of BHK cell extracts using an antiserum against a synthetic Pichinde peptide coupled to BSA

An antiserum to a synthetic Pichinde peptide coupled to BSA was prepared in rabbits. This antiserum was used to define the polypeptide encoded in the 3' proximal end of the S RNA. BHK cells were infected at a multiplicity of infection of 10 pfu/cell and labelled with L-[^{35}S] methionine at 22 to 25 hr post infection. Immunoprecipitation of infected cell lysates with the rabbit antiserum against the synthetic peptide coupled to BSA, resulted in the detection of the viral NP but not the GP-C (Fig. 21b). A few additional proteins were detected in this immunoprecipitation experiment; however, they appeared to be non-specific since the same proteins were detected in uninfected cell extracts immunoprecipitated with hamster anti-Pichinde serum (Fig. 21b). The viral NP was not detected in infected cell lysates immunoprecipitated with pre-immunization serum (Fig. 21b).

III. Discussion

The task of mapping the Pichinde viral genome was approached from three different levels of increasing resolution. Having shown that the viral genetic information resides on two RNA species (L and S), that the MWs of the L and S RNAs are 2.63 - 2.83 X 10^6 and 1.26 - 1.31 X 10^6 respectively and that the L and S RNAs contain different genetic information, one could deduce the potential coding capacity of each viral RNA species. Using this data, the 3 primary gene products, L, NP and pGP-C, can be assigned to the L or S RNA. Both NP and pGP-C can be mapped to the S RNA while the L protein is mapped to the L RNA. Due to the uncertainty in the MW of the L protein, it was also feasible that the L protein, NP and/or pGP-C mapped to the L RNA. This possibility was ruled out in the second approach when the recombinant virus, Re-2, was used to determine the gene assignment for the S RNA (Leung et al., 1981, Vezza et al., 1980; Compans et al., 1981; Harnish et al., 1983). Re-2 contains the S RNA of Munchique virus. The NP and GP-C of Re-2 were similar to that of wt Munchique virus as deduced by tryptic peptide mapping implying that both NP and pGP-C are encoded by the S RNA. Tryptic peptide analyses of the L proteins of Re-2, Pichinde and Munchique further substantiate the idea that the L protein is encoded by the L RNA (Harnish et al., 1983).

Since both NP and pGP-C have been unambiguously assigned to the S RNA, the next task was to determine the orientation of these two genes on the S RNA and to locate the gene(s) at the nucleotide sequence level. This involved the use of a synthetic peptide corresponding to the first 8 amino acids encoded in the 3' proximal end of the S RNA. The antiserum raised to this peptide coupled to an immunogen (BSA), was able to define the product encoded in the 3' proximal end of the S RNA as the NP. The 8 amino acid sequence encoded from residues 87 to 110 inclusive, represents the predicted amino terminus of the NP and seems to be able to elicit an humoral immune response in the rabbit. At least some of the antibodies generated is able to recognize the NP protein. The conformation of the native NP must therefore be such that the amino terminus consisting of at least 8 amino acids, is exposed at the surface of the molecule.

Since the antiserum to the coupled peptide immunoprecipitated the viral NP, this defines the initiation site of translation of the NP at the first UAC triplet (nucleotide residues 84-86) from the 3' terminus of the S RNA. This result is in agreement with the general model that the 5' proximal AUG functions as the initiator codon for most eukaryotic mRNAs (Kozak, 1983).

Assuming the simplest method of encoding the NP and pGP-C i.e. non-overlapping reading frames without splicing events, one could orient the two genes on the S RNA. The NP is encoded towards the 3' proximal end of the S RNA. By deduction, the pGP-C is encoded towards the 5' proximal end of the S RNA. Since the MW of the NP is 64,000, this protein should be encoded within a 1.9kb region of viral RNA starting at nucleotide 84 from the 3' terminus of the S RNA. The pGP-C whose MW is 42,000 should be encoded within a 1.3kb region of viral RNA towards the 5' terminus of the S RNA (Fig. 22). Since the S RNA is approximately 4kb and the amount of RNA required to code for both the NP and pGP-C is about 3.2kb, the remaining RNA could represent untranslated and intergenic regions. Sequencing studies of S cDNA clones have ruled out the possibility of overlapping

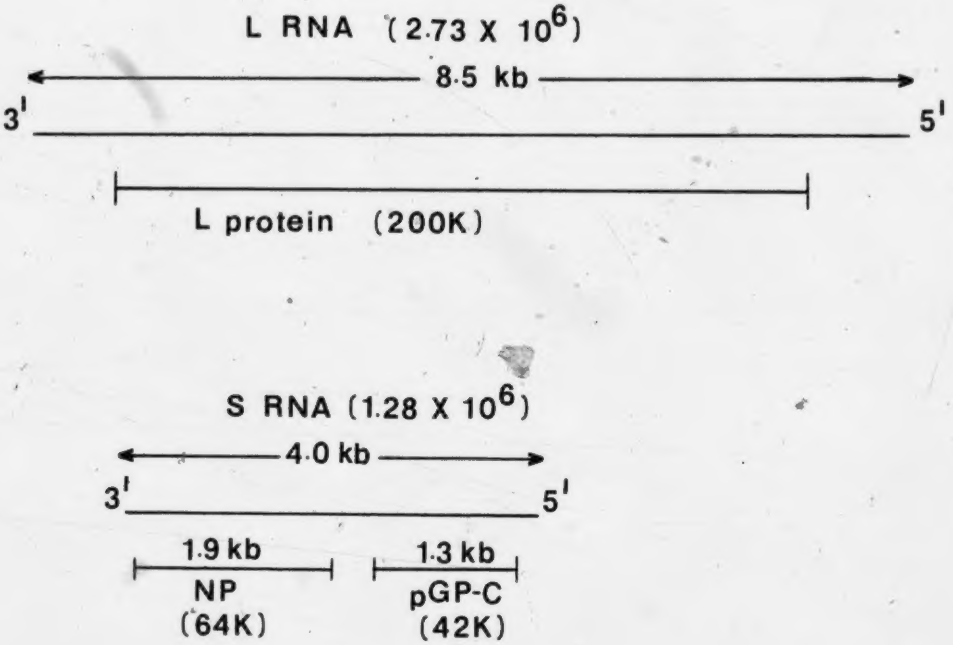

L RNA (2.73 X 10⁶)
8.5 kb
3'
5'
L protein (200K)
S RNA (1.28 X 10⁶)
4.0 kb
3'
5'
1.9 kb
NP
(64K)
1.3 kb
pGP-C
(42K)

Fig. 22. Mapping of the Pichinde viral genome.

reading frames in the S RNA (D. Bishop, personal communication).

The model in which the L RNA codes for the L protein and the S RNA codes for the NP and the pGP-C has the following implications. The NP and derivatives of GP-C i.e. GP1 and GP2, could provide the structural polypeptide components for the Pichinde virion. Since this virus is a negative-strand RNA virus, an RNA-transcription-replication function is required for propagation of the viral genome. An RNA transcriptase activity has been found associated with the Pichinde viral ribonucleoprotein complex (Leung _et_ _al_., 1977). This transcriptase/replicase activity may reside on the L protein. Our data show that the L protein is synthesized in relatively large amounts in infected BHK cells. Most of this protein seems to readily associate with the NP to form a complex of >100S, presumably a ribonucleoprotein complex. Very little free L protein is found in infected cells. The association of the L protein with the viral ribonucleoprotein complex supports the hypothesis that the transcriptase/replicase activity resides on the L protein. Definitive proof of the L protein being the transcriptase/replicase would require isolation of the L protein from the ribonucleoprotein complex and assaying for transcriptase and replicase activities _in_ _vitro_. This hypothesis could also be tested by using ts mutants. The ts mutants of Pichinde virus have been grouped into two recombination groups (Vezza and Bishop, 1977). It's likely that the ts lesion of one group resides on the L RNA while that of the second group resides on the S RNA. The prediction of this hypothesis is that the ts mutants of the first group should possess temperature sensitive transcription and replication functions. Moreover, the existence of the recombinant virus, Re-2, which contains the Pichinde L RNA and the Munchique S RNA suggests that the transcriptase/replicase encoded by the L RNA can also function in the transcription and replication of the S RNA from a different, yet closely related, strain of virus.

Cells persistently infected with arenaviruses will express the viral NP (Pedersen, 1979) implying that these cells must contain the S RNA. The viral genome does not appear to exist in the form of proviral DNA in persistently-infected cells (Rawls and Leung, 1979). A further prediction of the hypothesis that the L RNA encodes the transcriptase/replicase is that persistently-infected cells should also contain the L RNA since both transcriptase and replicase activities would be required for the expression of the viral NP.

These predictions can be tested when highly sensitive molecular probes are available to study the L and S RNAs of Pichinde virus.

CHAPTER 6

CONSTRUCTION OF RECOMBINANT cDNA PROBES FOR THE STUDIES OF GENOME STRUCTURE IN PICHINDE VIRUS

I. Introduction

Further studies on Pichinde virus and its replication in cultured cells require specific and sensitive molecular probes. Such probes would be useful in determining the nucleotide sequence relationships among the various viral RNA species i.e. L, S, 3.1kb and 1.9kb RNAs. These probes could also be used to analyze the intracellular sequence of events occurring during viral replication in vitro. Questions such as whether the messenger RNAs are polycistronic and/or monocistronic, whether there is preferential transcription of the L or S RNA and whether transcriptional controls operate in the regulation of virus replication can be addressed if molecular probes are available. Such probes could also be used to test the hypothesis that both the L and S RNAs are required to maintain viral persistence in vitro.

This chapter outlines the construction of molecular probes prepared by making cDNA clones from Pichinde viral RNA. These cDNA clones were used to study the nucleotide sequence relationships among the viral RNAs i.e. L, S, 3.1kb and 1.9kb RNAs.

II. Results

A. Identification of cDNA clones

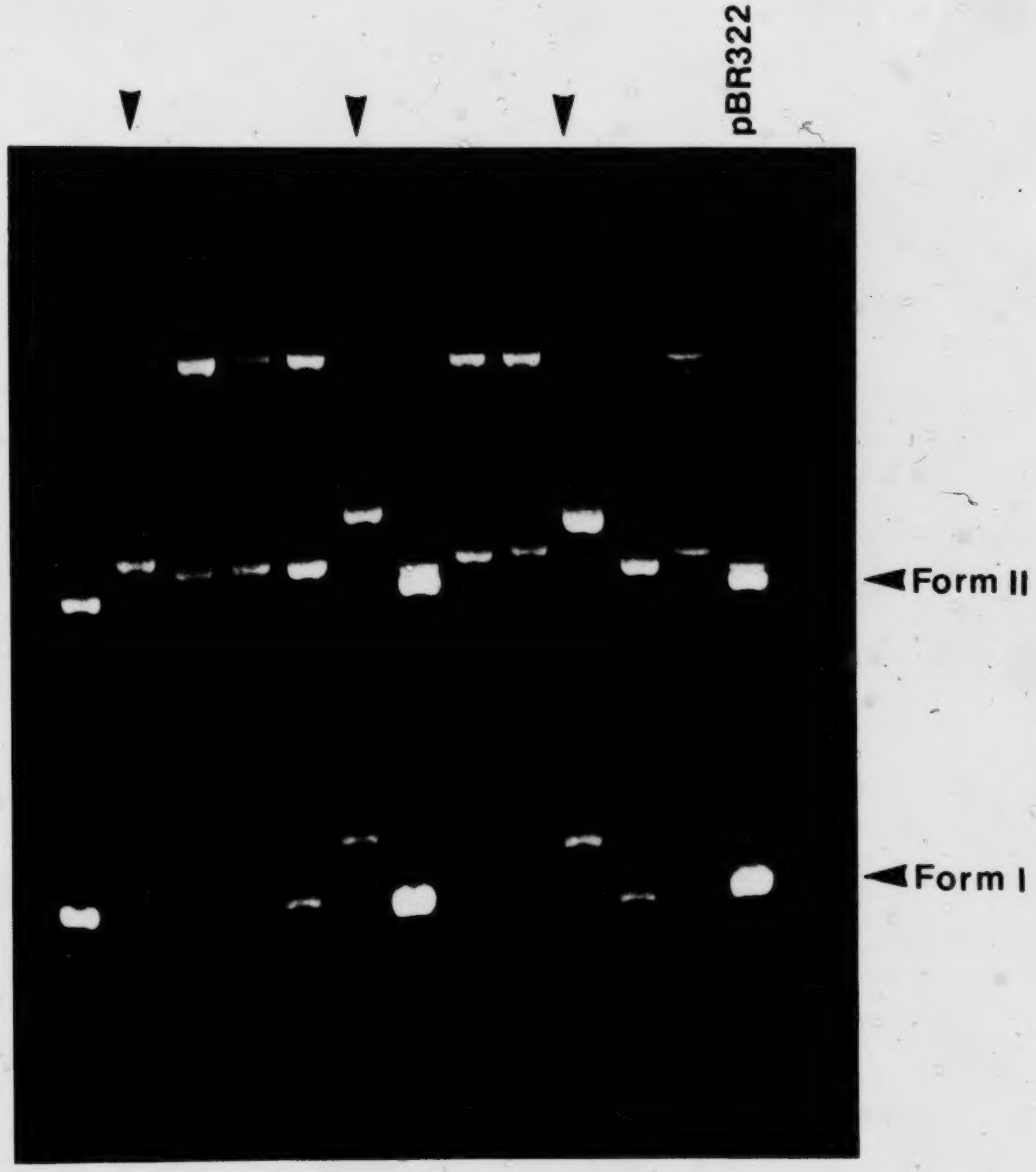
BHK-21 cells were infected at a multiplicity of 0.1 pfu/cell and virus was harvested at 48 to 72 hr post infection. The RNA profile of this purified virus preparation yielded 4 RNA species i.e. the virus-specific L and S RNAs and the cellular ribosomal RNAs, 28S and 18S. This RNA preparation was used to construct recombinant cDNA clones.

Bacterial colonies were initially detected by screening for antibiotic sensitivity. Since cDNA inserts had been cloned into the Pst 1 site of the ampicillin gene, transformants with the ampicillin-sensitive, tetracycline-resistant ($Ap^{S}Tc^{R}$) phenotype were selected. The plasmid DNAs of these transformants were purified according to the method of Birnboim and Doly (1979) and screened by size using agarose gel electrophoresis (Fig. 23). Transformants containing plasmid DNA larger than the vector pBR322 were selected for further characterization of their cloned inserts by sequence homology.

B. Determination of the specificity of the cDNA clones by hybridization studies

Since RNA (L, S, 28S, 18S) isolated from purified virus preparations was used to construct cDNA clones, one would expect the clones to segregate into two classes. One class of clones should contain virus-specific inserts i.e. L or S genetic information while the other class should contain ribosomal RNA-specific inserts. These two classes were differentiated by

Fig. 23. Screening of recombinant plasmid DNAs by size using agarose gel electrophoresis. Plasmid DNAs migrating slower than the vector pBR322 were selected as potential recombinant plasmids (designated by arrows).


pBR322
Form II
Form I

colony hybridization analyses. Clones were initially screened by colony hybridization. cDNA inserts of small size ($\leq$ 150bp) were however, not detected by this method. A more sensitive assay i.e. the dot hybridization assay was subsequently used for screening. Purified plasmid DNA was linearized and attached to nitrocellulose filters. Two identical filters were prepared. Filter A was probed with ^{32}P-labelled cDNA made from RNA extracted from purified Pichinde virus preparations (Fig. 24). Filter B was probed with ^{32}P-labelled cDNA made from ribosomal RNA extracted from uninfected BHK cells (Fig. 24). Positive hybridization signals with both probes indicate ribosomal RNA-specific inserts. A positive hybridization signal with Pichinde cDNA and a corresponding negative signal with ribosomal cDNA indicate a virus-specific insert. Seventeen of the twenty-one clones appear Pichinde-specific while four seem to be of ribosomal origin.

Analysis of the cloned cDNA inserts by Pst 1 digestion of the recombinant plasmid DNAs followed by agarose gel electrophoresis showed that the inserts of the Pichinde virus-specific clones ranged in size from 100bp to 1000bp with an average size of 300bp to 500bp (Fig. 25). DNA from the clone containing the largest insert yielded 2 digestion products implying that this cloned insert contains an internal Pst 1 site. Some of the recombinant DNA plasmids seemed to have lost one of the two Pst 1 sites since upon Pst 1 digestion, only linearized

Fig. 24. Identification of Pichinde virus-specific clones by dot hybridization. Filter A was probed with ^{32}P-labelled Pichinde cDNA while filter B was probed with ^{32}P-labelled BHK ribosomal cDNA.

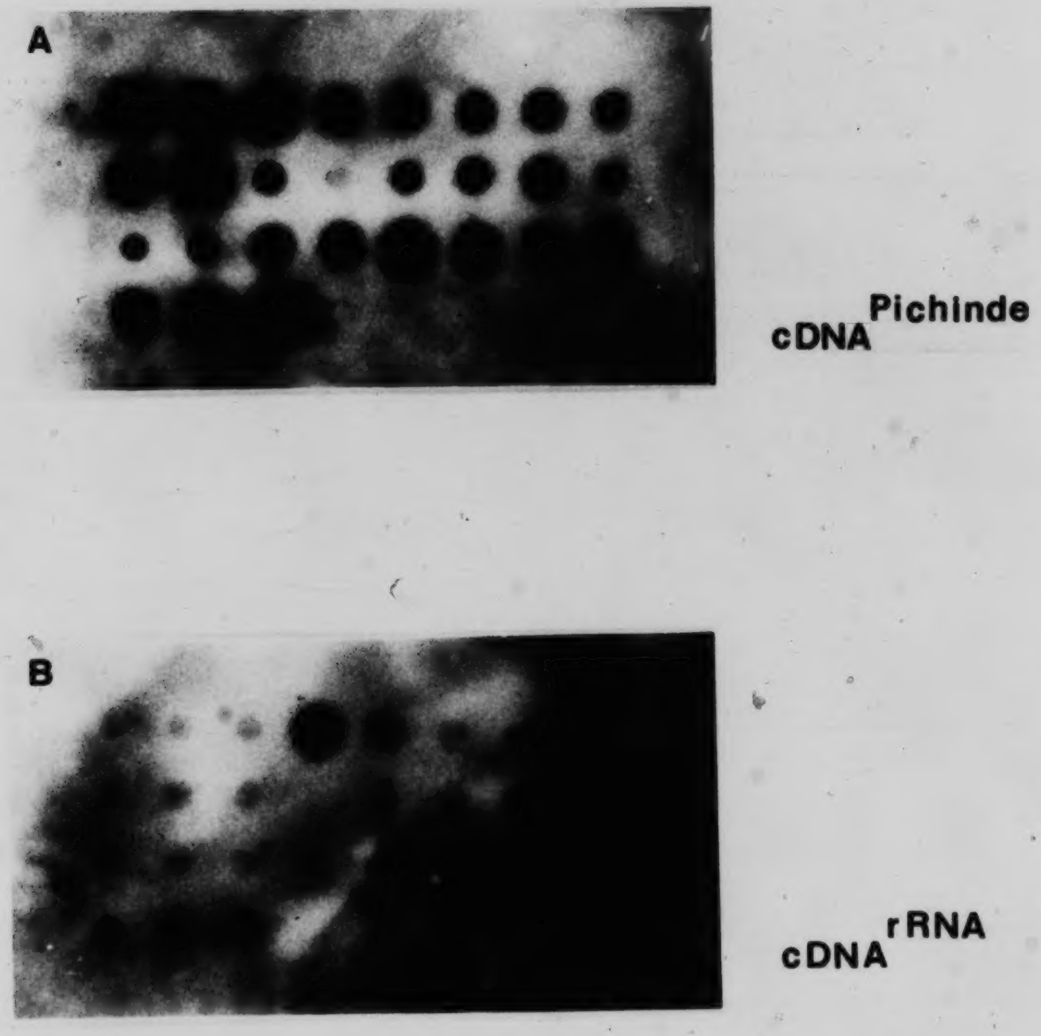
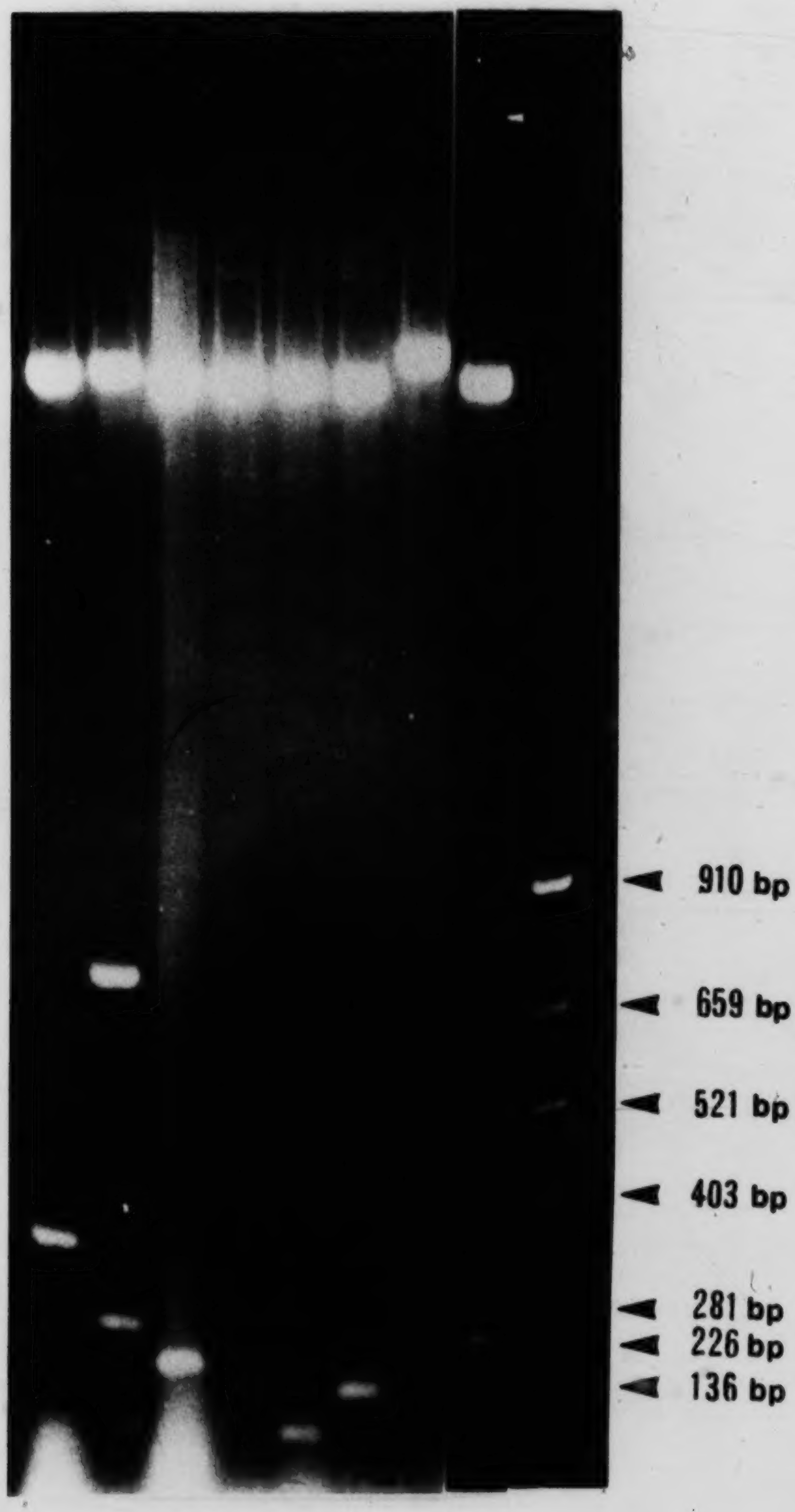

A
cDNA Pichinde
B
cDNA rRNA

Fig. 25. Agarose gel electrophoresis of Pst 1-digested recombinant plasmid DNAs. cDNA inserts ranged in size from 100 to 1000bp.


910 bp
659 bp
521 bp
403 bp
281 bp
226 bp
136 bp

plasmid DNAs, larger than linearized pBR322, were observed. The loss of a Pst 1 site from a recombinant plasmid may be attributed to contaminating nuclease activity during the preparation of the dG-tailed pBR322 vector.

C. Assignment of the Pichinde virus-specific cDNA clones to their appropriate genomic L or S RNA

Having identified the virus-specific clones, the next step was to assign the clones to their appropriate genomic L or S RNA. Two approaches were undertaken. ^{32}P-labelled cDNA probes, synthesized from L or S RNA fractionated by electrophoresis in agarose gel, were hybridized to the Pichinde-specific cloned DNAs in a dot hybridization (Table 9). Since the RNAs that were used to prepare the cDNA probes had been isolated from agarose gels after electrophoresis under denaturing conditions, one may expect the S RNA template to be contaminated with ribosomal RNA and degraded L RNA. Contamination of the S cDNA probe with ribosomal cDNA should not interfere with the interpretation of the results since only virus-specific cloned inserts were screened. Recombinant cDNA clones giving positive hybridization signals with L cDNA and negative signals with S cDNA were identified as being derived from the L RNA. Six L cDNA clones could be unambiguously identified in this assay. Similarly, recombinant cDNA clones giving positive hybridization signals with S cDNA and negative signals with L cDNA were identified as being derived from the S RNA. Three S cDNA clones could be identified in this assay. Plasmid DNA from clone 7 gave positive

TABLE 9

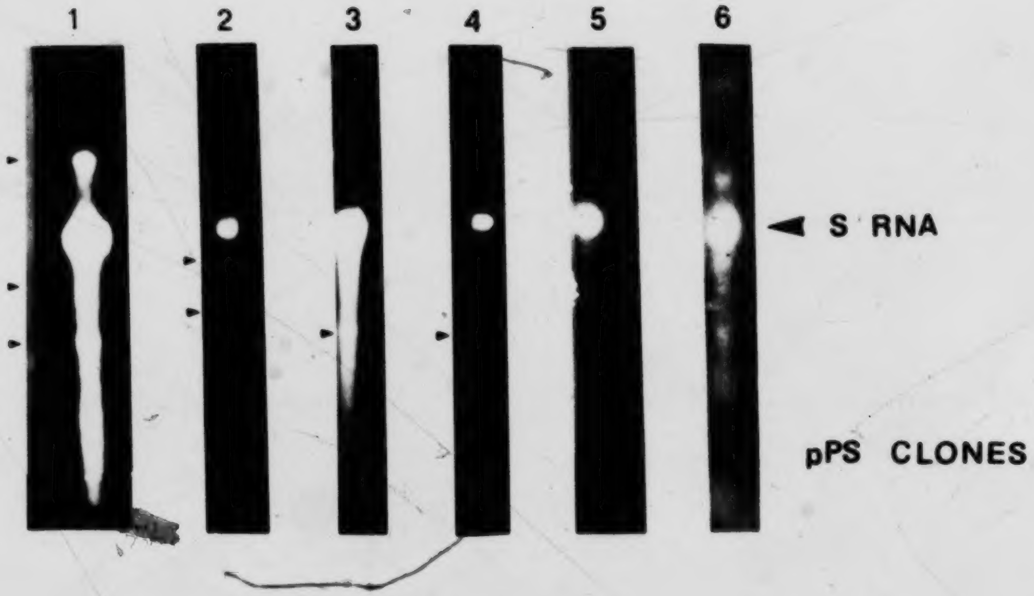
Assignment of the Pichinde virus-specific cDNA clones to their genomic RNAs (L or S) by dot hybridization using cDNA probes prepared from isolated L or S RNA

Clone number	Hybridization to cDNA from isolated L RNA	Hybridization to cDNA from isolated S RNA
1	+	-
2	+	-
3	-	+
4	-	+
5	-	-
6	-	-
7	+	+
8	-	+
9	-	-
10	+	-
11	-	-
12	-	-
13	-	-
14	+	-
15	+	-
16	-	-
17	+	-

hybridization signals with both probes. This clone was later shown to be an S cDNA clone by RNA blot analyses. Cross - hybridization of the L cDNA probe to the plasmid DNA from clone 7 indicates some sequence homology between the viral L and S RNAs. Although 17 Pichinde-specific cDNA clones had been initially identified, only 9 could be unambiguously assigned to their appropriate genomic RNA. A more sensitive approach was therefore necessary to assign all of the clones to the L or S RNA.

The second approach undertaken was analysis of the cloned DNAs by RNA blot analyses. If the Pichinde viral RNA preparation contained all 6 RNA species i.e. L, S, 28S, 18S, 3.1kb and 1.9kb RNAs, then one could simultaneously examine the nucleotide sequence relationships among the various RNAs. Thus, Pichinde virus was harvested from BHK cells 24 hr to 120 hr post infection after an input multiplicity of 1.0 pfu/cell and RNA was extracted from purified virus preparations. This RNA was electrophoresed in agarose gels, blotted onto nitrocellulose paper and probed with a nick-translated cloned cDNA probe (Fig. 26). The results of this study are summarized in Table 10. Six S cDNA clones were detected. Two of the cloned cDNAs (5, 9) hybridized to only the S RNA while four (3, 4, 7, 8) hybridized to the S RNA and the 1.9kb RNA. DNA from clones 4 and 7 also hybridized to the 3.1kb RNA but to a minor extent. DNA from clone 7 which hybridized to both S cDNA and L cDNA in the dot hybridization experiment, hybridized to 4 RNA species in the RNA blot experiment.

Fig. 26. Identification of L and S clones by RNA blot analyses. Hybridization of nick-translated plasmid DNA from pPS clones to the L, 3.1kb and 1.9kb RNAs is designated by arrows. Similarly, hybridization of nick-translated plasmid DNA from pPL clones to the S and 3.1kb RNA is designated by arrows.


1
2
3
4
5
6
S RNA
pPS CLONES

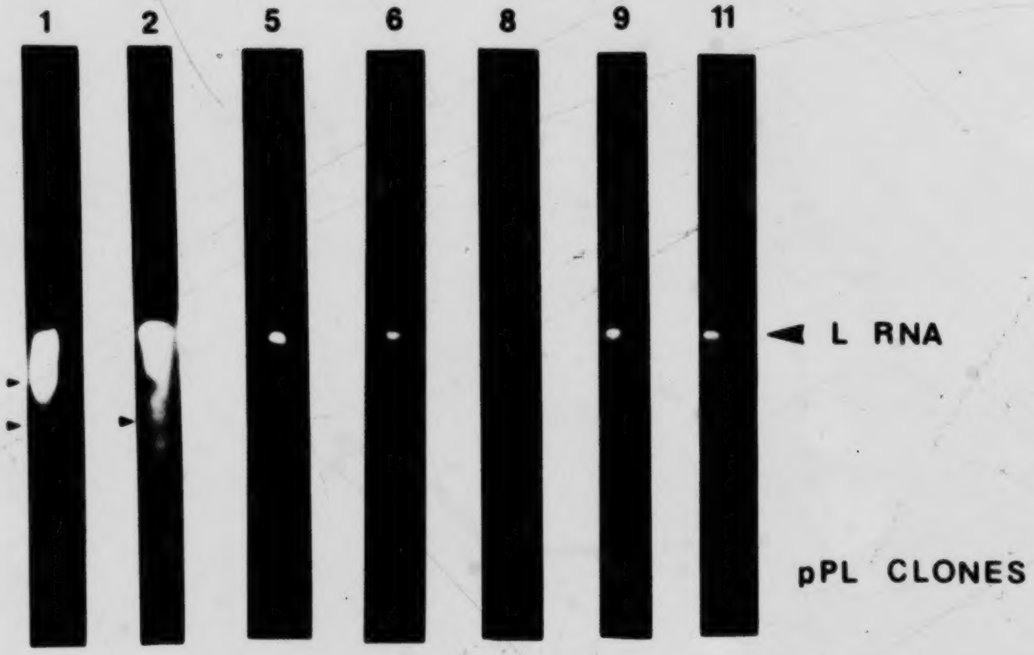

1
2
5
6
8
9
11
L RNA
pPL CLONES

TABLE 10

Summary of hybridization data generated by RNA blot analysis

Clone No.	S or L clone	Name	Hybridization to L RNA	S RNA	3.1kb RNA	1.9kb RNA
7	S-3' proximal	pPS1	++	++++	+	++
4	S-3' proximal	pPS2	-	++++	+	++
3	S-3' proximal	pPS3	-	++++	-	+
8	S-3' proximal	pPS4	-	++++	-	+
5	S	pPS5	-	++++	-	-
9	S	pPS6	-	++++	-	-
1	L	pPL1	++++	+++	+	-
2	L	pPL2	++++	?	+	-
16	L	pPL3	++++	++	-	-
17	L	pPL4	++++	++	-	-
6	L	pPL5	++++	-	-	-
10	L	pPL6	++++	-	-	-
11	L	pPL7	++++	-	-	-
12	L	pPL8	++++	-	-	-
13	L	pPL9	++++	-	-	-
14	L	pPL10	++++	-	-	-
15	L	pPL11	++++	-	-	-

Since the hybridization signal was strongest at the S RNA, clone 7 was designated an S clone. The 6 S clones were subsequently named pPS1 through pPS6 (p: plasmid vector pBR322; P: Pichinde; S: S RNA). The remaining 11 clones were assigned to the L RNA. Six cloned cDNAs hybridized to only the L RNA. Five cloned cDNA probes hybridized to both the S and L RNAs. Since the hybridization signals were strongest at the L RNA, these clones were designated L cDNA clones. DNA from cDNA clone 1 hybridized to 3 RNA species, L, S and 3.1kb while cDNA from clone 2 hybridized to 2 RNA species, L and 3.1kb. The 11 L cDNA clones were subsequently named pPL1 through 11 (p: plasmid vector pBR322; P:Pichinde; L: L RNA).

D. Orientation of the clones on the S RNA

cDNA clones that were derived from the 3' proximal end of the S RNA were identified in a dot hybridization using a 3' end-specific S RNA probe. The generation of this probe is outlined in Figure 27. RNA extracted from purified Pichinde virus preparations was labelled at its 3' termini with $[^{32}P]$pCp using RNA ligase. The S RNA was isolated after electrophoresis in agarose containing 10mM methylmercury hydroxide and subjected to controlled alkali digestion in 100mM Tris-HCl, pH9.5 at 95°C for 30 min. The resulting probe contained a mixture of 3' end-labelled S RNA fragments of varying lengths with the largest size class being approximately 6×10^5MW or 1.9kb which is 1/2 the length of the S RNA (Fig. 28). This probe was then used to

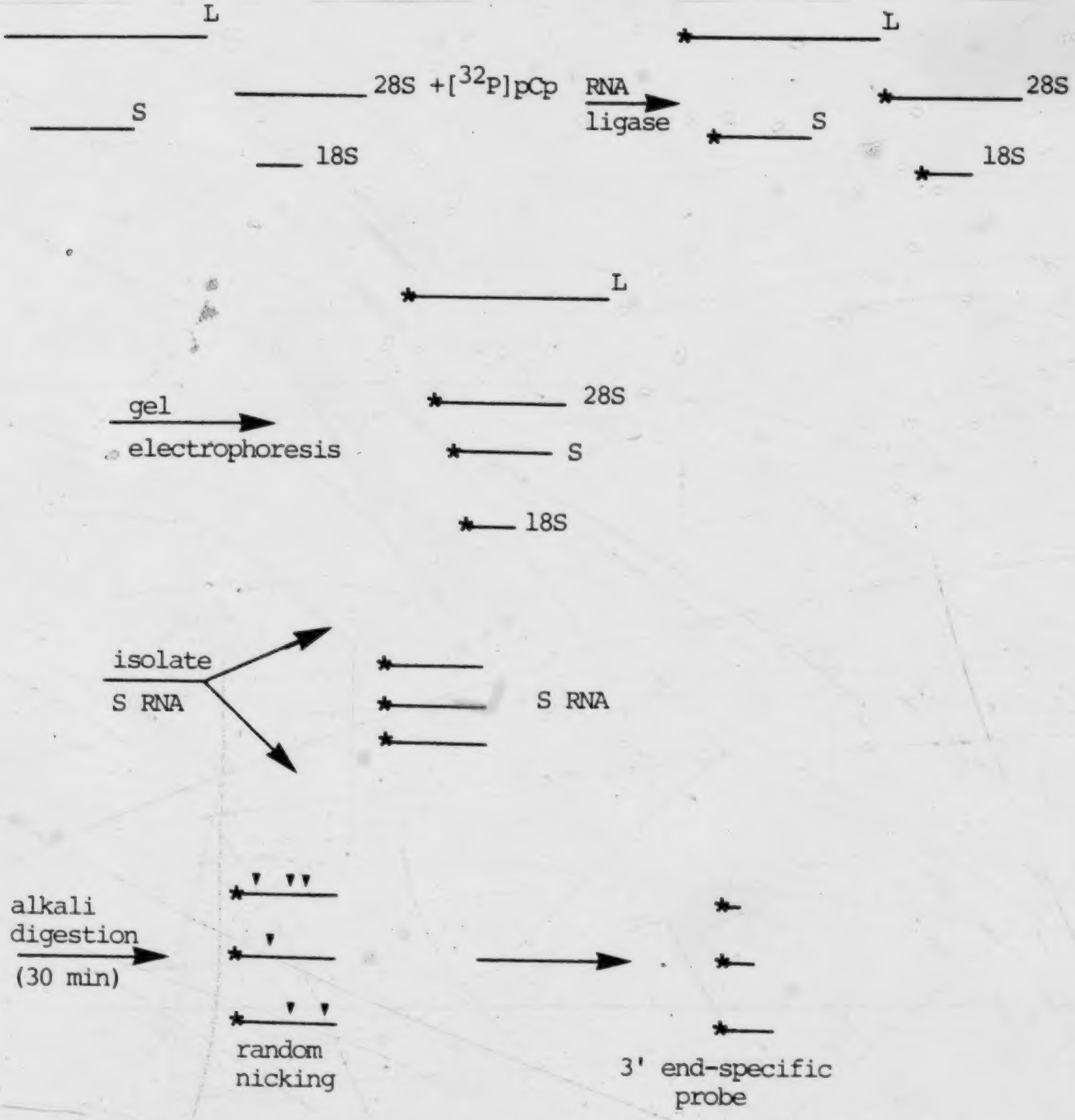
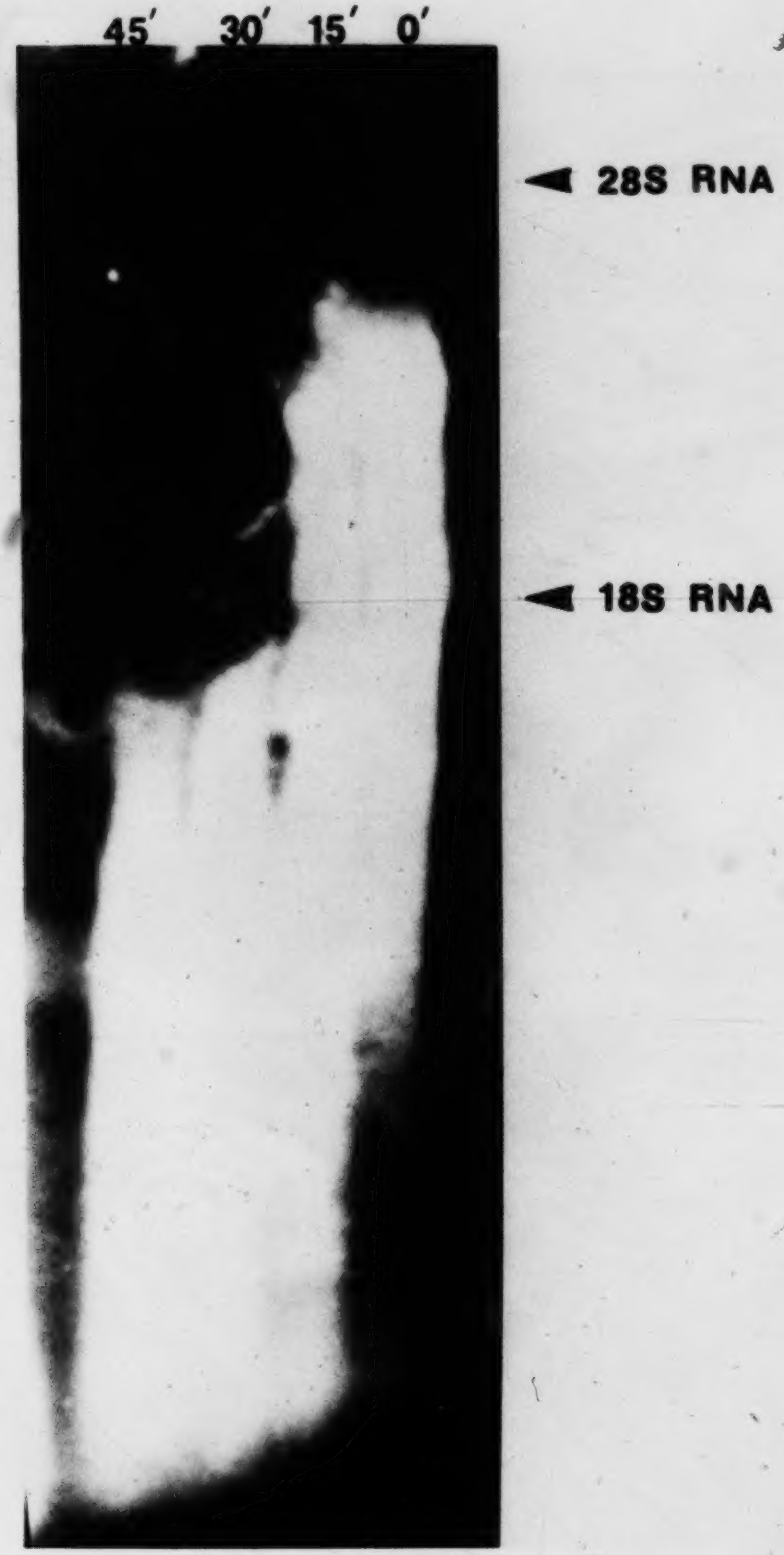

L
S
28S +[^{32}P]pCp
18S
RNA
ligase
L
28S
S
18S
gel
electrophoresis
L
28S
S
18S
isolate
S RNA
S RNA
alkali
digestion
(30 min)
random
nicking
3' end-specific
probe

Fig. 27. Generation of a 3' end-specific S RNA probe.

Fig. 28. Autoradiogram of 3' end-labelled S RNA preparations after alkali digestion for 0, 15, 30 and 45 min followed by electrophoresis in agarose containing 10mM methylmercury hydroxide.

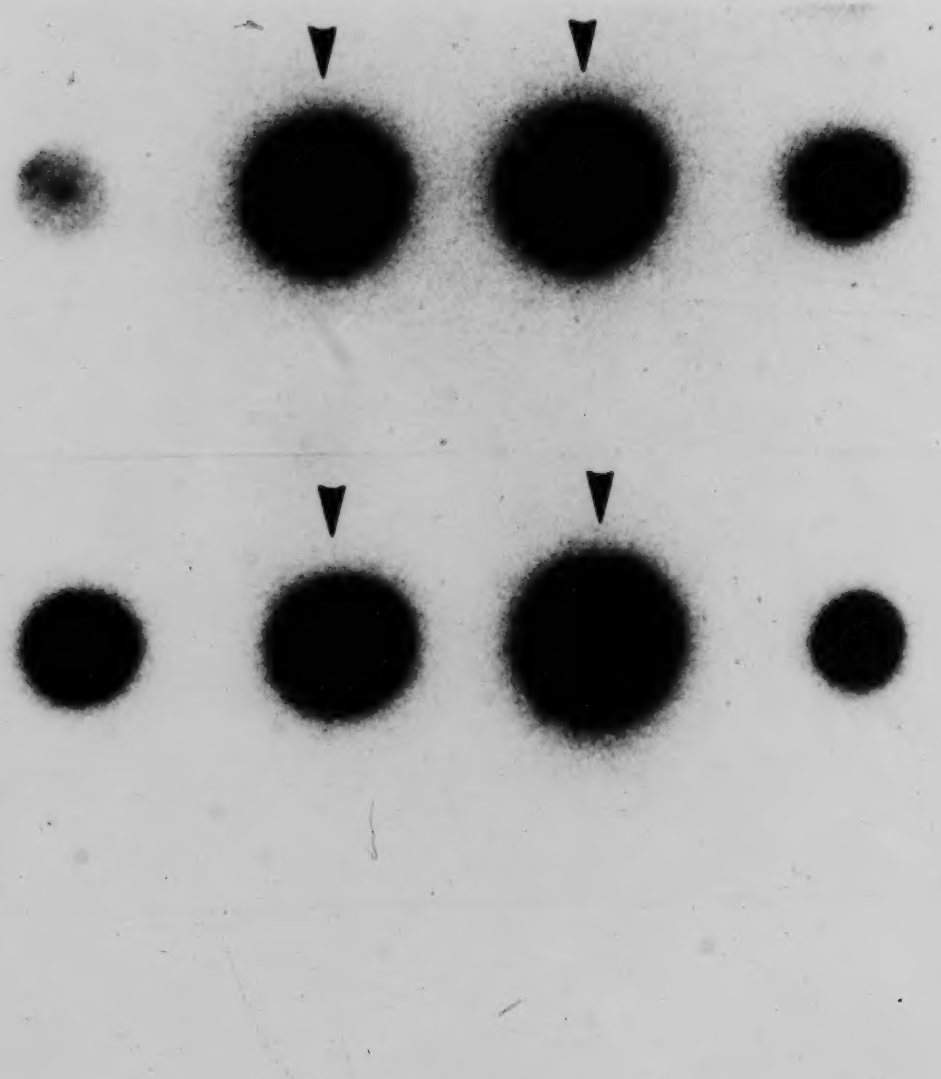

45′
30′
15′
0′
28S RNA
18S RNA

screen the recombinant plasmid cDNAs from the S clones in a dot hybridization where the plasmid cDNAs were linearized and attached to nitrocellulose filters (Fig. 29). DNA from four clones (pPS1 to pPS4) hybridized strongly to the 3' end-labelled S RNA probe. DNA from the remaining 2 S cDNA clones (pPS5, pPS6) did not hybridize to the 3' end-labelled S RNA probe.

III. Discussion

Highly sensitive and specific molecular probes are necessary to obtain a better understanding of the biology of Pichinde virus. This chapter outlines the construction of molecular probes in the form of cDNA clones to analyze the nucleotide sequence relationships among the viral RNAs i.e. L, S, 3.1kb and 1.9kb RNAs. Pichinde cDNA clones were prepared by using random DNA primers and reverse transcriptase to transcribe RNA extracted from purified virus preparations. The second strand of DNA was synthesized using the Klenow fragment of E. coli DNA polymerase 1. The double-stranded DNA was then cloned into the Pst 1 site of pBR322. Since the genetic information of Pichinde virus appears to reside in the L and S RNAs, the emphasis was on obtaining cDNA clones specific to these two RNAs. The template RNA for cDNA synthesis was therefore obtained from virus preparations that contained mainly the 4 RNA species i.e. L, S, 28S and 18S RNAs. The resulting cDNA clones should segregate into 2 classes i.e. virus-specific clones containing L or S genetic information and ribosomal-specific

Fig. 29. Identification of S clones that map within the 3' proximal end of the S RNA by dot hybridization. A 3' end-specific S RNA probe was hybridized to linearized plasmid DNA on a nitrocellulose filter. Four clones were detected (arrows) that mapped within the 3' proximal end of the S RNA.



clones. Since the 28S and 18S ribosomal RNAs constitute up to 50% of the large molecular weight RNAs present in purified virus preparations (Carter et al., 1973), one would expect approximately 50% of the cDNA clones to be of ribosomal RNA origin. Analyses of the cloned DNAs by dot hybridization using Pichinde cDNA probes and ribosomal cDNA probes showed that out of 21 cDNA clones, 4 were of ribosomal RNA origin while 17 were of viral RNA (L or S) origin. The preponderance of virus-specific clones may imply preferential transcription of the L and S RNAs by reverse transcriptase which could be a reflection of the secondary structure of the various RNAs. The G + C content of the ribosomal RNAs is considerably higher than that of the L and S RNAs (Carter et al., 1973). Using the conditions described for cDNA synthesis, the L and S RNAs may have been more readily denatured than the ribosomal RNAs thereby allowing preferential transcription of the virus-specific RNAs.

Of the 17 Pichinde virus-specific cDNA clones, 11 were derived from the L RNA and 6 were derived from the S RNA. Using a 3' end-specific S RNA probe to screen the recombinant plasmids of the S cDNA clones in a dot hybridization, 4 cDNA clones (pPS1 to pPS4) were identified as being derived from the 3' proximal end of the S RNA. The probe consisted of a mixture of 3' end-labelled S RNA fragments of varying lengths with the largest size class being 1.9kb. Therefore, the conclusion is that the inserts from cDNA clones pPS1 to pPS4 map within a

1.9kb region of the S RNA at the 3' terminus. These inserts are derived from various parts of the 1.9kb region of the S RNA since the hybridization profiles, obtained by RNA blot analyses of viral RNA probed with nick-translated cloned DNAs, are different. Two clones, pPS5 and pPS6, appear to map outside of the 1.9kb region since plasmid DNA from these 2 clones did not hybridize to the 3' end-specific S RNA probe.

The L and S clones were used to probe the nucleotide sequence relationships among the viral RNAs, L, S, 3.1kb and 1.9kb. This was accomplished by RNA blot analyses of viral RNA preparations containing L, S, 28S, 18S, 3.1kb and 1.9kb RNAs. These RNA preparations were probed with nick-translated cloned cDNAs. Plasmid DNA from clones pPS1 to pPS4 identified in addition to the S RNA, the 1.9kb RNA. No other cloned DNA detected the 1.9kb RNA. Since clones pPS1 to pPS4 are derived from the 3' proximal end of the S RNA, this suggests that there is sequence homology between the 1.9kb RNA and the 3' proximal end of the S RNA. The cloned inserts of pPS1 through pPS4 represent a total of approximately 2kb of DNA. Since these inserts are derived from different regions within the 3' proximal end of the S RNA, this suggests that the sequence homology between the 1.9kb RNA and the 3' proximal end of the S RNA is extensive. The 1.9kb RNA may thus represent a truncated S RNA species.

RNA blot analyses also show that there is some sequence homology between the L and S RNAs. Plasmid DNA from clone pPS1 cross-hybridized with the L RNA while plasmid DNAs from clones pPL1, pPL3 and pPL4 cross-hybridized with the S RNA. Liquid hybridization studies have shown that although the L and S RNAs are mainly unique, they do share some common sequences (Leung *et al*., 1981). RNA sequencing studies have shown that Pichinde L and S RNAs share 90% sequence homology at their 3' termini for the first 19 nucleotides (Auperin *et al*., 1982). From nucleotides 20 to 50, the L and S RNAs share 50% sequence homology. The nucleotide sequences are then expected to diverge at the start of the coding region since the L and S RNAs contain different genetic information. Plasmid DNA from clone pPS1 cross-hybridized with the L RNA and the 1.9kb RNA. Since the 1.9kb RNA seems to be derived from the 3' proximal end of the S RNA and the 3' termini of the L and S RNAs share some common sequences, then it seems likely that the cDNA insert of clone pPS1 maps at the extreme 3' terminus of the S RNA. The L and S RNAs also seem to share some common internal nucleotide sequences since plasmid DNAs from cDNA clones pPL1, pPL3 and pPL4 cross-hybridized with the S RNA but not with the 1.9kb RNA.

Plasmid DNAs from cDNA clones pPS1, pPS2, pPL1 and pPL2 also cross-hybridized with the 3.1kb RNA. This suggests that all 4 RNA species, L, S, 3.1kb and 1.9kb, share some common sequences internally and at their 3' termini. The origin of the 3.1kb RNA is however unclear.

Both the 1.9kb and 3.1kb RNAs seem to be produced late in the infection cycle. If the 1.9kb RNA is indeed a truncated S RNA, derived from the 3' proximal end of the S RNA, the 1.9kb RNA would be sufficient to encode the nucleoprotein gene. This RNA could be generated via 2 possible mechanisms (Fig. 30). The negative-sense genomic S RNA is transcribed into a full-length positive-sense template from which progeny negative-sense genomic S RNAs are made. Late in the infection cycle, the 1.9kb RNA is made via a splicing mechanism or an internal initiation mechanism. These two possibilities can be readily differentiated by comparing the nucleotide sequences at the 3' and 5' termini of the 1.9kb RNA with that of the S RNA. If the 1.9kb RNA is in fact the NP gene, then this represents gene amplification late in the infection cycle. Amplification of the NP gene may represent a control mechanism that is involved in the regulation of Pichinde viral gene expression.

The origin of the 3.1kb RNA is, as yet, unclear. This RNA species shares some common nucleotide sequences with the L and S RNAs and is produced late in the infection cycle. The 3.1kb RNA may be of viral origin i.e. derived from the L or S RNAs or of cellular origin. These possibilities may be differentiated by cloning the 3.1kb RNA and using the cloned DNAs to probe sequence homology in the L and S RNAs and in cellular genes.

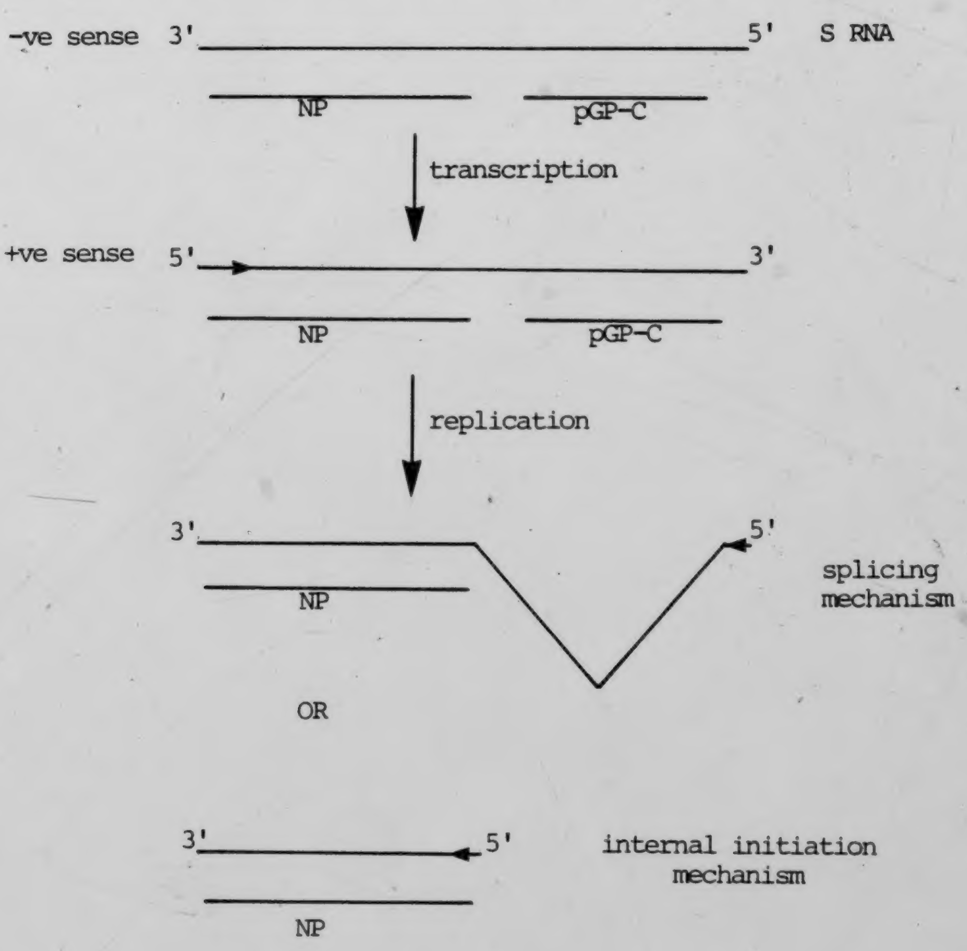

-ve sense
3'
5'
S RNA
NP
pGP-C
transcription
+ve sense
5'
3'
NP
pGP-C
replication
3'
5'
NP
splicing mechanism
OR
3'
5'
internal initiation mechanism
NP

Fig. 30. Generation of the 1.9kb RNA via two possible mechanisms.

CHAPTER 7

SUMMARY

The aim of this thesis project was to define the Pichinde viral genes and their corresponding gene products. The major observations were as follows:

1. The genetic information of Pichinde virus resides in a minimum of 2 RNA species i.e L and S. Late in infection or after a high multiplicity of infection, 2 additional RNA species designated 3.1kb and 1.9kb were detected.

2. The MW estimates of the L and S RNAs were 2.63-2.83 X 10^6 and 1.26-1.31 X 10^6 respectively.

3. The L and S RNAs were shown to be mainly unique by liquid hybridization studies using cDNA probes made from isolated L and S RNAs.

4. Based on the potential coding capacity data of the L and S RNAs, the hypothesis that the L RNA encodes the L protein while the S RNA encodes both the NP and pGP-C was developed.

5. The NP gene was mapped at the 3' proximal end of the S RNA and the initiation site of translation was defined at nucleotide residues 84-86 i.e at the first UAC triplet from the 3' terminus of the S RNA.

6. Molecular probes in the form of L and S cDNA clones were constructed and used to show that there is some sequence homology among all 4 RNA species i.e L, S, 3.1kb and 1.9kb. In addition, the 1.9kb RNA shares extensive sequence homology with the 3' proximal half of the

S RNA.

Further work is required to determine the exact nature of the 1.9kb and 3.1kb RNAs. The 1.9kb RNA may represent a truncated S RNA species that contains the information for the NP. It may also represent a mRNA species or a replicative-intermediate form. These possibilities may be differentiated by determining the sense (+ or -) of the 1.9kb RNA. The origin of the 3.1kb RNA is, as yet, unclear. This RNA species may be of viral or of cellular origin. A clone of the 3.1kb RNA would be useful in distinguishing between these two possibilities.

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